In vitro studies on human hematopoiesis

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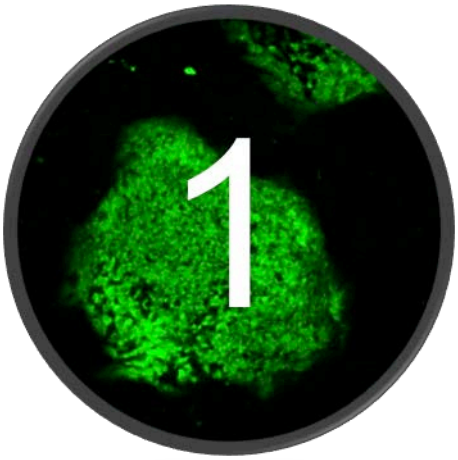
Abbreviations

AGM	Aorta-gonado mesonephros
AHR	Aryl hydrocarbon receptor
BAC	Bacterial Artificial Chromosome
BM	Bone marrow
BMP	Bone morphogeneic protein
CAR	Chimeric antigen receptor
CAS	CRISPR associated nuclease
CB	Cord blood
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DN	Double negative
DP	Double positive
DSB	Double strand break
E-	Embryonic
EB	Embryoid Body
eGFP	Enhanced green fluorescent protein
EMP	Erythro-myeloid precursors
EpiSC	Epiblast stem cells
ERG	Ets related gene
ESC	Embryonic stem cell
Etv	Ets variant gene
FL	Fetal liver
Flt	Fms-like tyrosine kinase
Fos	v-FOS FBJ murine osteosarcoma viral oncogene homolog

FRT	Flippase recognition target
Gata	GATA zing finger domain-containing protein
GCM	Goblet cell metaplasia
Gfi	Growth factor-independent
GMP	Granulocyte-monocyte precursor
GVHD	Graft-versus-host disease
hESC	Human embryonic stem cell
hIPSC	Human induced pluripotent stem cell
HLA	Human leukocyte antigen
HOX	Homeobox
HPC	Hematopoietic progenitor cell
HR	Homologous Recombination
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem or progenitor cell
IBD	Inflammatory bowel disease
IEL	Intra-epithelial lymphocytes
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
IL-xR	Interleukin x receptor
indel	Insertion of deletion
IPSC	Induced pluripotent stem cell
ISP	Immature single positive
IT-HSC	Intermediate-term repopulating HSC
KIR	Killer cell immunoglobulin-like receptors
Klf	Kruppel-like factor
LIF	Leukemia inhibitory factor
Lin	Lineage
Lmo	Lim domain only
LMP	Lympho-myeloid precursor
LoxP	Locus of X-over P1
LSK	$Lin^{-}Sca1^{+}cKit^{+}$
LT-HSC	Long-term repopulating HSC

Lti	Lymphoid tissue inducer
LTR	Long terminal repeats
MEF	Murine embryonic fibroblast
Meg	Megakaryocyte
Meis	Meis homeobox protein
MEP	Megakaryocyte-erythrocyte precursor
mESC	Murine embryonic stem cell
MHC	Major histcompatibility complex
miPSC	Murine induced pluripotent stem cell
MLP	Myelo-lymphoid progenitor
MPP	Multipotent progenitor population
MPS	Mononuclear phagocyte system
mRNA	Messenger Ribonucleic acid
Myb	v-MYB avian myeloblastosis viral oncogene homolog
Myc	v-MYC avian myelocytomatosis viral oncogene homolog
NCR	Natural cytotoxity receptors
NHEJ	Non Homologous End Joining
NK	Natural killer
Oct	Octamer-binding proteins
PAMP	Pathogen-associated molecular pattern
PAS	Para-aortic splanchnopleura
Pbx	Pre B-cell leukemia transcription factor
Prdm	PR domain containing protein
PRR	Pattern recognition receptor
PSC	Pluripotent stem cell
ROR	RAR-related orphan receptor
Run	Runt-related transcription factor
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SIN	Self-inactivating
SLT	Secondary lymphoid tissue

Sox	SRY (sex determining region Y)-box
SRC	SCID repopulating cell
SSEA	Stage specific embryonic antigen
ST-HSC	Short-term repopulating HSC
TALEN	Transcription activation like effector nuclease
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
TGF	Transforming growth factor
TH	T helper
TNF	Tumor necrosis factor
TRA	Tumor rejection antigen
tracRNA	trans-activating CRISPR RNA
UCB	Umbilical cord blood
UG	Uro-genital
VEGF	Vascular endothelial growth factor
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
Wnt	Wingless-type MMTV integration site family
YS	Yolk sac
ZFN	Zinc finger nuclease
Zfp	Zinc finger protein



Part I :

In vitro generation of hematopoietic cells from pluripotent stem cells

The use of transgenic transgenic MYB-eGFP hESC as a study tool for in vitro hematopoiesis

1. Summary of part I

For the treatment of severe hematopoietic diseases such as severe combined immunodeficiency (SCID) or Wiskott-Aldrich syndrome (WAS), current therapy involves allogeneic transplantation with hematopoietic stem cells (HSC) from a matched sibling (haploidentical donor). Alternatively gene corrected autologous HSC can be used, a method called gene therapy. This gene correction currently involves transduction of HSC with viral vectors, which encode a corrected copy of the affected gene. Here, the corrected gene is inserted in the genome *at random* and is constitutively expressed. While this therapy has proven successful in the clinic, adverse events have been described due to insertion of the viral vector in, or in the proximity of, potential oncogenes leading to malignant transformation. Recent advances in transgenic methods have provided tools to edit a specific genomic sequence, so called “tailored nucleases”. This allows the correction of the mutated gene itself, without the need for random integration of a corrected copy of the gene. While a proof of principle of this method has been recently reported, genetic correction using this method has low efficiency and would only be feasible in cases where the corrected gene confers a selective advantage over the mutated copy. In addition, as currently no protocols are available to culture hematopoietic stem cells, there are thus no means to control for mutations or random insertions induced by this method prior to transplantation.

Pluripotent stem cells (PSC) have the ability to generate every cell type of the human body. Almost a decade ago, a protocol was described to confer this property to differentiated cells isolated from adult skin cells. This process is called “reprograming”. Recently also cells isolated through less invasive methods, such as blood cells, have been successfully reprogrammed. The cells generated using this method are called induced pluripotent stem cells (iPSC). These cells can be cultured indefinitely without losing their pluripotency, and can thus be cultured in a clonal manner. As genetic modification using tailored nucleases is readily implemented in PSC research and their properties allow thorough screening for off-target effects and selection of successfully modified cells, these cells would be the ideal candidate to generate gene corrected HSC for gene therapy.

The generation of HSC from PSC has been the goal of many stem cell researchers. Although several reports have claimed the generation of cells with HSC characteristics from human embryonic stem cells (hESC), another type of PSC, no reports convincingly show the generation of *bona fide* HSC.

During *in vivo* embryonic development, also other progenitor cells are generated besides HSC. These progenitor cells are largely generated early in development in the yolk sac (YS) before the generation of a HSC. Together, the progeny of these cells contains cells of all blood cell lineages.

We therefore set out to define whether HSC are generated upon *in vitro* differentiation of hESC. To this end, we inserted a transgenic marker into hESC, which allows to track the emergence and presence of HSC. This transgenic marker induces expression of a fluorescent protein encoding gene (enhanced green fluorescent protein, eGFP) when expression of a key HSC marker is induced. As key HSC marker, we chose MYB, as this gene was shown to be highly expressed in murine HSC and MYB was found to be essential for establishing a hematopoietic system based on HSC in the mouse model.

Using an *in vitro* hematopoietic differentiation protocol for hESC, we were able to show that during the onset of hematopoiesis, no MYB-eGFP expression could be detected in cells that had emerged from hemogenic endothelium. Only later on in culture, $CD34^+$ cells expressing MYB-eGFP became apparent. Upon assessment of the phenotype and properties of these MYB-$eGFP^+$ cells, we found that while these express several progenitor markers, they were also marked by expression of myeloid lineage markers. Upon analysis of the lineage potential of MYB-$eGFP^+$ progenitors, these cells showed clear myeloid granulocytic lineage restriction. These data thus argue against the formation of MYB-$eGFP^+CD34^+$ HSC in this *in vitro* differentiation system. In addition, the myeloid cells that are generated from hESC upon hematopoietic differentiation resemble myeloid cells generated in the embryo during a wave of HSC independent hematopoiesis in the YS.

In conclusion, based on our study and previous reports, we conclude the *in vitro* hESC hematopoiesis resembles yolk sac hematopoiesis and thus that there is no formal proof for the generation of MYB-$eGFP^+$ *bona fide* HSC *in vitro*. However, use

of a transgenic reporter line, as the one described here, might aid in the optimization of protocols with the aim of generating HSC from PSC.

2. Samenvatting van deel I

Ernstige bloedziekten zoals *severe combined immunodeficiency* (SCID) of *Wiskott-Aldrich syndrome* (WAS) worden momenteel behandeld door middel van een allogene transplantatie met bloedstamcellen (hematopoietische stamcellen, HSC) van een compatibel familielid (haploidentisch donor). Een alternatieve behandeling bestaat uit transplantatie van autologe HSC waarin het aangetaste gen werd gecorrigeerd, de zogenaamde gentherapie. Deze genetische correctie gebeurt door het inbrengen van virale vectoren in de HSC. Deze virale vectoren bevatten een correcte kopie van het aangetaste gen. Bij deze methode wordt een correcte kopie van het gen willekeurig in het genoom ingebracht en ononderbroken tot expressie gebracht. Deze therapie wordt momenteel succesvol toegepast in de kliniek, ondanks maligne transformatie die beschreven werd bij deze methode. Vaak ontstaan deze ongewenste effecten door de integratie van de virale vectoren in, of nabij, potentiële oncogenen. Deze inserties kunnen mogelijk leiden tot maligne transformatie en de ontwikkeling van leukemie induceren. Door recente ontwikkelingen in transgene methoden, is het momenteel mogelijk om een specifieke genomische sequentie aan te passen, door gebruik van "*tailored nucleases*". Dit laat toe het gemuteerde gen zelf te corrigeren, zonder willekeurig inbrengen van een gecorrigeerde kopie. Een bewijs voor de toepasbaarheid van dit principe werd recent beschreven voor gentherapie in autologe HSC. Echter, de efficiëntie van deze methode is laag en zou enkel succesvol kunnen zijn in gevallen waar het gecorrigeerde gen een sterk selectief voordeel geeft ten opzichte van het gemuteerde gen. Momenteel zijn geen methodes beschikbaar om HSC in kweek te houden, dus is het niet mogelijk om na te gaan of deze methode ongewenste mutaties of additionale integraties van het gecorrigeerde gen induceerde in de cellen.

Pluripotente stamcellen (PSC) hebben de mogelijkheid om elk celtype van het lichaam te vormen. Bijna 10 jaar geleden, werd een methode beschreven om deze eigenschappen over te brengen naar cellen die geïsoleerd werden uit de huid van volwassen individuen. Dit proces noemt men "reprogrammering". Recent werden ook methoden beschreven om cellen die geïsoleerd zijn met behulp van minder invasieve methoden, zoals bloedcellen, te reprogrammeren. De cellen die via deze methode worden gegenereerd noemt men geïnduceerde pluripotente stamcellen (iPSC). Deze

cellen kunnen ongelimiteerd worden gekweekt zonder hun pluripotente eigenschappen te verliezen, en kunnen dus op een klonale manier worden opgegroeid. Genetische modificatie met *tailored nucleases* word reeds toegepast in PSC onderzoek. Deze cellen zouden ideaal zijn voor de generatie van HSC voor gentherapie. Gezien hun eigenschappen, laten deze cellen immers toe om na te gaan of er ongewenste effecten zijn door de behandeling. Bovendien kunnen correct aangepaste cellen geselecteerd worden.

De generatie van HSC uit PSC is al lang een doel voor stamcelonderzoekers. De succesvolle generatie van cellen met HSC eigenschappen uit humane embryonale stamcellen (hESC), dewelke een ander soort pluripotente stamcel is, werd reeds geopperd. Echter, geen enkele publicatie kon overtuigend de aanwezigheid van cellen die aan alle HSC eigenschappen voldoen aantonen. Tijdens embryonale ontwikkeling worden ook verschillende progenitorcellen gegenereerd naast HSC, dewelke niet aan alle stamcel eigenschappen voldoen. Deze progenitoren worden in het embryo vooral gegenereerd in de dooierzak vóór het ontstaan van een HSC. De dochtercellen die door deze verschillende progenitoren worden gegenereerd, bevatten samen alle types bloedcellen.

In onze studie hebben we onderzocht of HSC worden gevormd tijdens de *in vitro* differentiatie van hESC. Hiervoor werd een transgene merker ingebouwd in hESC die toelaat om de vorming en aanwezigheid van HSC te volgen. Deze transgene merker breng een fluorescent eiwit (eGFP) tot expressie, gelijktijdig met de expressie van een belangrijke HSC merker. Als HSC merker kozen we MYB, gezien dit gen sterk tot expressie komt in muis HSC en het MYB eiwit essentieel is om een hematopoietisch systeem op te bouwen op basis van HSC in de muis.

Door gebruik te maken van een methode voor *in vitro* hematopoietisch differentiatie van hESC, waren we in staat om aan te tonen dat tijdens bloedvorming, geen MYB-eGFP expressie kon worden gedetecteerd in cellen die ontstaan uit bloedvormend endotheel. Pas later tijdens de kweek konden $CD34^+$ cellen worden gedetecteerd die positief waren voor MYB-eGFP. Tijdens het bepalen van het fenotype en de multipotente eigenschappen van deze cellen, vonden we dat ondanks de expressie van verschillende progenitor merkers, deze cellen ook positief waren voor myeloide merkers. Bij het bestuderen van het lineage potentieel, werd duidelijk dat deze cellen niet multipotent zijn, maar enkel in staat zijn om myeloide granulocytaire cellen te

vormen. Deze data ondersteunen de hypothese dat MYB-eGFP$^+$ CD34$^+$ HSC worden gevormd bijgevolg niet. Bovendien vertonen de myeloide cellen die worden gevormd tijdens *in vitro* differentiate van hESC sterke gelijkenissen met myeloide cellen die worden gevormd in de dooierzak van het embryo onafhankelijk van HSC.

We concluderen, dat op basis van onze studie en voorgaande publicaties, *in vitro* hESC hematopoiese lijkt op dooierzak hematopoiese en dat er dus geen formeel bewijs is voor de vorming van echte HSC *in vitro*. Het gebruik van transgene reporterlijnen, zoals deze hier beschreven, zou kunnen helpen in de verbetering van methoden die richten op de vorming van HSC uit PSC.

3. *Introduction of part I*

3.1 Pluripotent stem cells

Pluripotent stem cells (PSC) can give rise to cells of all three germ layers (endoderm, mesoderm and ectoderm) and thus possess the ability to generate virtually every cell type of the human body.

After decades of research on teratocarcinoma cells (1, 2) and embryonal carcinoma cells (3, 4), murine embryonic stem cells (mESC) were first derived in 1981 (5, 6). mESC are derived from the inner cell mass (ICM) of a developing blastocyst, through culture on a mitotically inactivated fibroblast feeder layer (Figure 1).The first human embryonic stem cell (hESC) lines were derived by the group of James Thomson 17 years later (7). These hESC are derived by culture of human pre-implantation embryos and develop through a post-ICM intermediate (PICMI) (Figure1) (8). Both murine and human ESC are cultured on a feeder layer, which consists of murine embryonic fibroblasts (MEF). However, feeder-free culture systems for pluripotent stem cells (PSC) have been described (9, 10). Both mESC and hESC can be kept in culture indefinitely without differentiation, using the aforementioned culture techniques.

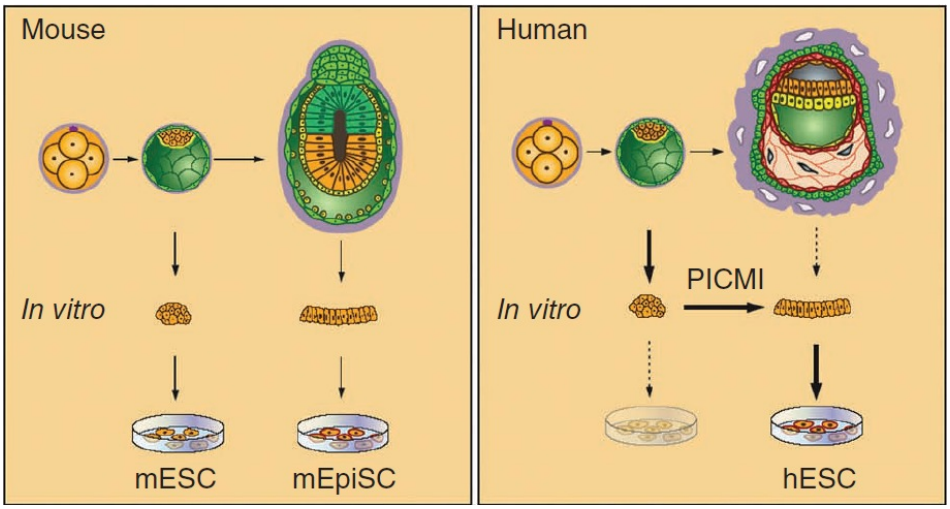


Figure 1: *Differences in the origin of murine embryonic stem cells (mESC) and human embryonic stem cells (hESC). Derivation of hESC proceeds through a post-inner cells mass (PICMI) stage, leading to epiblast-like stem cells (EpiSC). In contrast, mESC can be isolated directly from the ICM (figure adapted from O'leary et al. (8)).*

Both murine and human ESC are pluripotent, however they are markedly different in their origin and characteristics. While mESC are ICM derived, hESC show close similarity to epiblast-like stem cells (EpiSC)(Figure1) (11, 12). These differences are reflected in the different behavior of hESC compared to mESC: they are not dependent on LIF signaling, and readily undergo apoptosis upon dissociation (13-15). The surface marker profile of mESC and hESC was also found to be different (table I) (16). mESC are in a naive pluripotent ground state, while EpiSC are in a primed state, meaning that their fate is somewhat skewed, rendering them not fully pluripotent *sensu stricto*. PSC with similar characteristics as hESC have been derived from mouse post implantation blastocysts (11). These EpiSC do not efficiently contribute to blastocyst chimeras, proving their priming (11, 17). During differentiation into post-implantation epiblast cells, naive PSC are skewed to become primed PSC, with more restricted differentiation capacity, and elicit different sensitivity to environmental cues (18). It has been shown that EpiSC can be successfully reprogrammed towards naive PSC in the murine system (19); similarly hESC have been induced towards a naive pluripotent state by addition of kinase inhibitors (20)

Table I. Surface marker profile of human and murine ESC (data adapted from Ginis et al.(16)).

Surface marker	Human ES (H1)	Murine ES (D3)
SSEA1	-	+
SSEA3	+	+
SSEA4	+	-
TRA1-60	+	-
TRA1-81	+	-

SSEA: stage specific surface antigen; TRA: tumor rejection antigen

A standard assay to assess pluripotency of PSC is the ability to generate teratomas upon injection of mESC and hESC in immune deficient mice (6, 7). Teratomas, which are formed after injection of ESC, should contain cells derived from all three germ layers, which proves the pluripotent properties of ESC. mESC pluripotency is evaluated in the tetraploid complementation assay. In this assay, tetraploid blastocysts are injected with mESC (21, 22). The ESC derived germ cells should then be able to give rise to a healthy adult animal.

The derivation and use of hESC lines remains a topic of ethical debate and their use in the clinic is limited. Almost a decade ago the group of Shinya Yamanaka described protocols which allowed the induction of pluripotent stem cell (PSC) properties in fully differentiated adult cells. The cells generated through this method are called “induced pluripotent stem cells” (iPSC). Both murine iPSC (miPSC) and human iPSC (hiPSC) were initially generated by reprogramming of fibroblasts (23, 24). In these reports, reprograming was obtained by introducing *Oct3/4*, *Sox2*, *c-Myc* and *Klf4* through retroviral transduction of the somatic cells. iPS cells show close morphological similarity to ESC and have similar properties (23-25). For murine iPSC it was shown that these can contribute to the development of a chimeric murine embryo after blastocyst injection, formally proving their pluripotency (26, 27).

Since these initial reports, other protocols have been described to generate hiPSC from different sources which are less invasive, such as hematopoietic stem cells (HSC) from cord blood (CB) samples or peripheral blood mononuclear cells (PBMC) (28-30). Also, advances have been made in reprogramming using non-integrating techniques such as transfection of mRNA (31), transfection of protein (32) or transduction with non-integrating Sendai viral particles (33). All these methods circumvent the use of potentially oncogenic viral integration techniques and bring the application hiPSC in the clinic one step closer.

3.2 Human embryonic stem cell transgenesis

The generation of transgenic models has become the standard method to study the function of genes. In the murine system, the use of knockout models or reporter strains has greatly added to the knowledge of gene function during hematopoietic development. Similar *in vitro* models are powerful tools to study human hematopoiesis. In addition, reporter cell lines can be used for the optimization of *in vitro* protocols that aim at generating HSC.

Transgenic mice are frequently generated by bacterial artificial chromosome (BAC) transgenesis. BACs are large plasmids containing genomic DNA of the organism of interest. For the murine and also for the human genome a library has been generated with an average insert size of approximately 200kbp (34, 35). These BACs can be modified in bacteria through recombineering (36, 37). This allows for the induction of point mutations and introduction of deletions, selection cassettes, genetic code for fluorescent markers as well as recombination sites (LoxP, FRT). In addition, BACs can be used for the introduction of homology arms in donor constructs for homologous recombination (HR) in the endogenous genomic locus (38). Alternatively, as promoter regions and regulatory elements are often included within the BAC sequence, modified BACs can be used for the generation of reporter cell lines (39, 40). Insertion of such large genomic sequence can isolate the transgene, so that expression is not influenced by neighboring genes (41).

To date, only a limited set of hESC reporter lines are available, which are useful in the analysis of hematopoietic development. The group of Andrew Elefanty has generated hESC reporter lines which mark primitive streak mesoderm (MIXL1) (42), β-globin expression (43), hemogenic endothelium and emerging HSCs (SOX17) and hemogenic endothelium and hematopoietic cells (RUNX1) (meeting proceedings and personal communication).

It has to be noted that although murine transgenic protocols are well established, protocols for transgenic modification of hESC have only improved in recent years. One of the major limitations has been the induction of apoptosis upon dissociation of hESC (15). This hampers transfection of hESC by electroporation and nucleofection for which a suspension of single cell hESC is required (44, 45). This problem was

partially alleviated by blocking the pathway leading to apoptosis through addition of the Rho kinase inhibitor Y-27632 (14).

An additional problem with hESC transgenesis is the low efficiency of successful HR. HR is essential if reporter constructs are to be integrated in their endogenous loci. While reports have shown feasibility of this method in hESC using “gene trap” vectors, in which the expression of a selectable marker is driven by the gene to be targeted (42, 46), HR in hESC remains a relatively rare event (47, 48).

With the emergence of tailored nucleases the efficiency of targeted transgenesis has improved. The use of tailored nucleases has proven to be highly efficient for the introduction of locus specific mutations (49-51). These nucleases are all based on a similar concept: nuclease proteins are guided to a specific genomic sequence to be targeted. This induces a sequence specific double strand break (DSB) in the DNA (Figure 2). There are 2 options by which a cell will subsequently repair DSB. Either the cell will ligate the two ends of the DSB, in a process called non-homologous end joining (NHEJ). This process generally introduces mutations by insertions or deletions of one or more nucleotides (indel mutations). NHEJ can be used to generate knockout cell lines. Alternatively, the cell may use a homologous DNA strand as a template for repair, in the process of homology directed repair (HDR). HDR can used to introduce transgene insertions by cotransfection of an excess of donor plasmid (52).

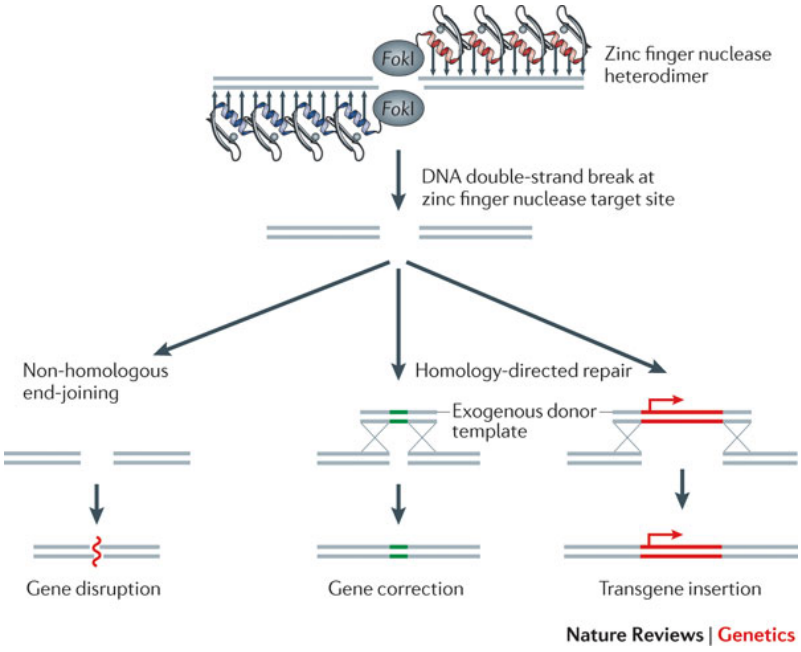


Figure 2: *Options for genome repair upon induction of double strand breaks (DSB) by tailored nucleases.*

Induced DSB are corrected by one of two mechanisms. Either the cellular repair machinery ligates both ends of the break together through the process of non-homologous end-joining (NHEJ), or the break is repaired through homology directed repair (HDR). The latter method can be used to introduce transgenes (Figure adapted from Naldini et al. (52)).

Different tailored nucleases have been described: zinc finger nucleases (ZFN), transcription activator- like effector nucleases (TALEN) and Clustered Regulatory Interspaced Short Palindromic Repeats and associated nucleases (CRISPR/Cas9) (Figure 3). ZFNs consist of a pair of heterodimeric FokI nuclease domains, which are linked to a tandem of zinc finger domains. Each domain recognizes a nucleotide triplet in the genomic sequence. These zinc finger domains confer sequence specificity to the nuclease activity. ZFN generally consist of 6-8 zinc finger domains, that recognize 18-24 base pairs, which should in theory be sufficient to generate a site specific DSB. TALEN are based on a similar principle as ZFN, but have the advantage that single base recognition can be used in their design, in contrast to the triplet design of ZFN. The CRISPR/Cas9 system uses an alternative method for DNA recognition. Here a sequence specific crispr RNA (crRNA) anneals to a trans-

activating crispr RNA (tracRNA), this complex is then bound by the Cas9 nuclease and guided to the targeted DNA sequence (reviewed by Gaj *et al.* (53)). The specificity of the CRISPR/Cas9 system can be further improved by the use of modified Cas9 proteins (nickases) or modified guiding RNA (50). The use of these tailored nucleases greatly increases the amount of potential HR events.

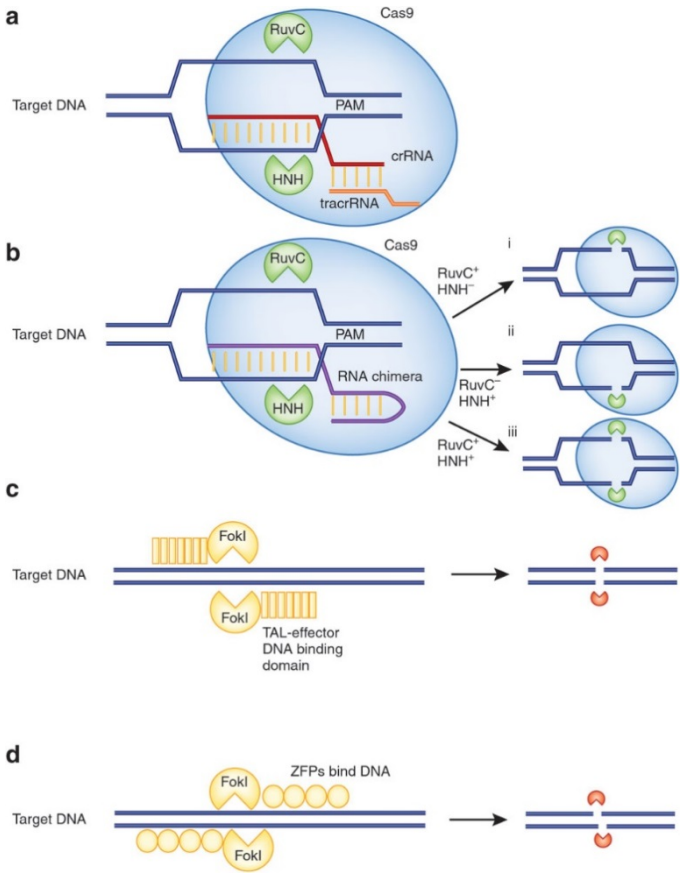


Figure 3: *Different options for genetic engineering using tailored nucleases.*

Several methods for the introduction of DNA breaks have been described, either a CRISPR/Cas9 complex can be used, in which a tracRNA can be introduced to induce sequence specific DNA breaks(a) or a chimera of the 3' crRNA end and the 5' tracrRNA end can be used (b). Alternatively, heterodimeric FokI nuclease domains can be coupled to either (c) transcription activator- like effectors (TALE) or (d) zinc finger (ZF) motifs (d), which confer sequence specificity to these nucleases (Figure adapted from Barrangou et al. (54)).

3.3 Hematopoiesis

During murine hematopoiesis, hematopoietic cells with different characteristics are generated during the development of the embryo, at different locations and different time points (Figure 4)(reviewed by Dzierzak and Speck (55)). Hematopoiesis occurs both inside the embryo and in extra-embryonic tissues and consists of different waves. The first wave of hematopoiesis is termed the “primitive” wave and is initiated outside the embryo proper, within the yolk sac (YS). The second wave of hematopoiesis is termed the “definitive” wave and occurs both outside the embryo proper and inside the embryo. Definitive hematopoiesis is initiated in the YS and shifts towards hematopoietic sites inside the embryo. Here, HSC are generated in the aorta-gonado-mesonephros (AGM)/splanchnopleura region, which will subsequently migrate to the fetal liver (FL). In the FL, these HSC mature and expand, after which they colonize the bone marrow to sustain lifelong hematopoiesis.

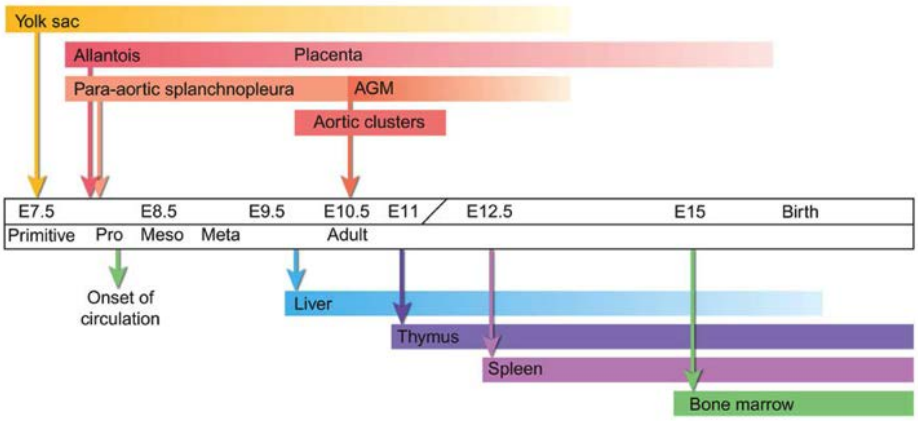


***Figure 4**: Overview of the ontogeny of the hematopoietic system in mice.*

Sites of active hematopoiesis are shown at the respective time points during embryonic development. E: embryonic day, AGM: aorta-gonado-mesonephros (Figure adapted from Dzierzak et al. (55))

3.4 Primitive hematopoiesis

During the primitive hematopoietic wave, the first hematopoietic cells are formed in the extra-embryonic tissues around E7.5. These cells are formed within the blood islands of the YS (56). Here, hematopoietic cells are found in close proximity of the endothelial cells lining these blood islands. The first blood cells generated within the blood islands are nucleated red blood cells and primitive macrophages. They are marked by their large size and the expression of both embryonic and fetal hemoglobin chains (57). During primitive hematopoiesis no HSC are formed, as at E7.5-E8.25 no transplantable HSC can be derived from the YS. At this timepoint, no evidence for the presence of multipotent progenitors can be found (58).

3.5 Early definitive hematopoiesis

The primitive wave of hematopoiesis is followed by a definitive wave, spatially overlapping both YS and AGM/splanchnopleura regions. During early definitive hematopoiesis, the YS gives rise to multiple types of hematopoietic (progenitor) cells. At E7.5-E8.5 monocytes are generated, which are part of the mononuclear phagocyte system (MPS) in the YS. These monocytes are generated independent of a HSC and sustain lifelong. The cells generated at this timepoint include tissue macrophages such as Kupffer cells, skin Langerhans cells and microglia. The YS has also been shown to form a lympho-myeloid restricted precursor (LMP) already before the initiation of AGM hematopoiesis. It has been reported recently, that lymphoid potential can be found through *ex vivo* culture of the YS and para-aortic-splanchno pleura (PAS) endothelium derived at E9.5 (59). At this timepoint HSC have not yet been formed. Proof for this concept *in vivo* has also been provided recently (60).

3.6 Late definitive hematopoiesis

After the initial wave of definitive hematopoiesis in the YS, *bona fide* HSC are formed in the aorta-gonado-mesonephros (AGM)/splanchnopleura region. Proof for the generation of HSC in the AGM region at E10 was provided almost two decades ago (58). Using explant cultures of YS, AGM and FL regions, it was shown that HSC formation is initiated in the E10 AGM region, and that the YS lacks any transplantable activity at this time point. Immature HSC are formed in the ventral region of the E10 AGM directly from the endothelial lining, a concept also found in other model organisms, such as the zebrafish (61, 62). However, HSC generated in the AGM do not immediately hold their full potential, but first require functional maturation and expansion in the FL (63). These mature definitive HSC will then seed the spleen and bone marrow to sustain lifelong HSC-dependent hematopoiesis. The embryo becomes dependent on HSC hematopoiesis around E15.

Human hematopoiesis has been described to follow similar pathway (Figure 5)(reviewed by Peters *et al.* (64)).

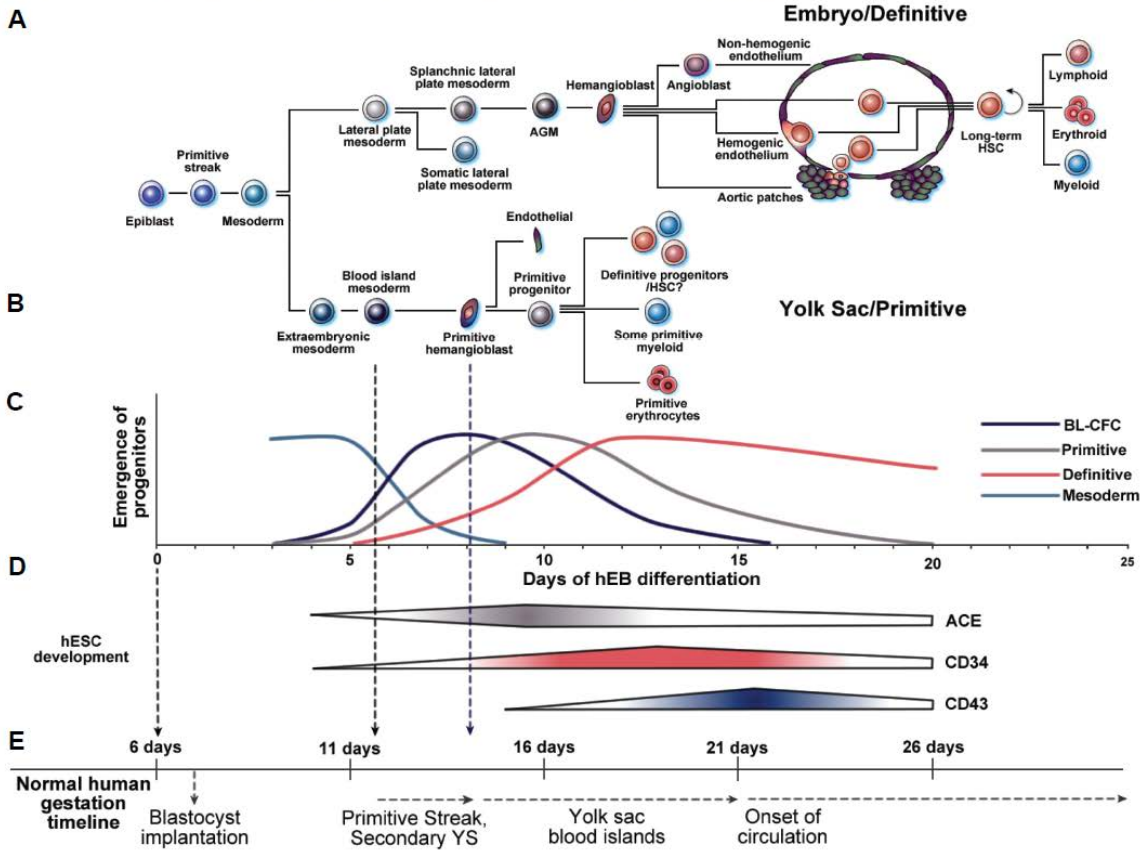


***Figure 5**: Overview of human hematopoietic development.*

During human hematopoiesis, the embryo will generate a primitive hemangioblast from the extraembryonic mesoderm and generate primitive hematopoietic progenitors (15-18 days of gestation). In the AGM region, the hemogenic endothelium will give rise to HSC (21-26 days of gestation)(Figure adapted from Peters et al.(64))

During the onset of hematopoiesis hematopoiesis, several transcription factors are important at different time points. Some of these transcription factors are already essential during the onset of primitive hematopoiesis, while others are not essential until the hematopoietic system becomes dependent on HSC. Among these factors are SCL, LMO2, GATA2, RUNX1 and MYB.

During early hematopoiesis SCL is an important factor, as mice lacking this transcription factor die around day 9.5 of gestation (E9.5). In the yolk sac of these $SCL^{-/-}$ animals, no hematopoietic activity can be detected (65). Similarly $LMO2^{-/-}$ mice fail to engage primitive and definitive hematopoiesis, this genotype is embryonically lethal around E9.75 (66). Other factors are not essential for the onset of primitive hematopoiesis, both are necessary for HSC generation and expansion. One of these factors is GATA2. As primitive and early definitive hematopoiesis is initiated, these mice survive until the point where the embryo generates and expands HSC. These $GATA2^{-/-}$ mice die around E10.5 (67). $RUNX1^{-/-}$ mice, die at E12.5. While erythrocytes have been described to be abnormal, suggesting a role already in primitive hematopoiesis, these mice do generate primitive erythrocytes (68). Using conditional deletion models, Chen et al. were able to show that RUNX1 plays an essential role during endothelial to hematopoietic cell transition, but not once the HSC are formed in the AGM (69). During definitive hematopoiesis, the transcription factor MYB has been shown to play an essential role in regulating HSC. in the murine system, a $MYB^{-/-}$ transgene is lethal E15 (61). This is due to a failure of the embryo to switch to HSC based hematopoiesis. More recently, conditional MYB knockout models were described, where the important role of MYB in HSC biology has been emphasized. Not only is MYB highly expressed in long-term repopulating HSC (LT-HSC), it plays an essential role in the maintenance of self-renewal properties (62, 63).

There seems to be a differential role of MYB during early and late definitive hematopoiesis. The first is HSC independent while the latter depends on HSC. Here recent reports show that tissue macrophages develop from MYB- YS precursors (64). This data suggests that MYB is a preferred marker for HSC, as it can distinguish HSC based hematopoiesis from primitive and early definitive hematopoiesis.

3.7 Hematopoietic stem cells

A HSC has three essential characteristics: 1) it is capable of generating every type of blood cell (*multipotent*); 2) is able to reconstitute the blood system of animals upon transplantation (*engraftable*); and 3) is able to divide, with one of the daughter cells maintaining these properties (*self-renewing*). A cell that lacks any one of these three properties is not a *bona fide* HSC, but rather a type of hematopoietic precursor.

The characterization of *bona fide* HSC using surface markers is of great interest for both the clinic and fundamental research. As a number of distinct multipotent stages are generated from the LT-HSC, the differential surface expression of membrane markers should be able to discriminate between these stages of HSC development (Figure 6). The HSC population can be functionally subdivided into LT-HSC, intermediate-term repopulating HSC (IT-HSC) and short-term repopulating HSC (ST-HSC). Eventually, the ST-HSC will differentiate into a population of multipotent progenitors (MPP). All of these cells are multipotent and provide (limited) engraftment, however only the LT-HSC can sustain lifelong hematopoiesis. The MPP will undergo lineage commitment during further differentiation. In the human system, the MPP develops either into a myelo-lymphoid progenitor (MLP) or a common myeloid progenitor (CMP). The MLP can give rise to B, T, NK, dendritic and monocytic cells, while the CMP can develop into a granulocyte-monocyte precursor (GMP) or a megakaryocyte-erythrocyte precursor (MEP) that will differentiate into granulocytes and monocytes and megakaryocytes and erythrocytes, respectively (reviewed by Doulatov *et al.* (70)).

The surface phenotype of both murine and human LT-HSC has been partly characterized (Figure 6). All murine HSC reside within the lineage negative (Lin^-) $Sca1^+cKit^+$ (LSK) population. Here, the MPP can be excluded, by their expression of

CD34 and their absence of CD150 expression. The LT-HSC containing population can be defined as being $Lin^{-}CD34^{-}CD150^{+}CD48^{-}CD41^{-}Flt3^{-}CD49b^{lo}$. This extended phenotype defines the LT-HSC population, as about 33-50% of cells are LT-HSC. For human HSC, use of the $Lin^{-}CD34^{+}CD38^{-}CD45RA^{-}Thy1^{+}Rho^{lo}CD49f^{+}$ phenotype described by Notta *et al*. defines a population in which about 14-28% of the cells are LT-HSC (71).

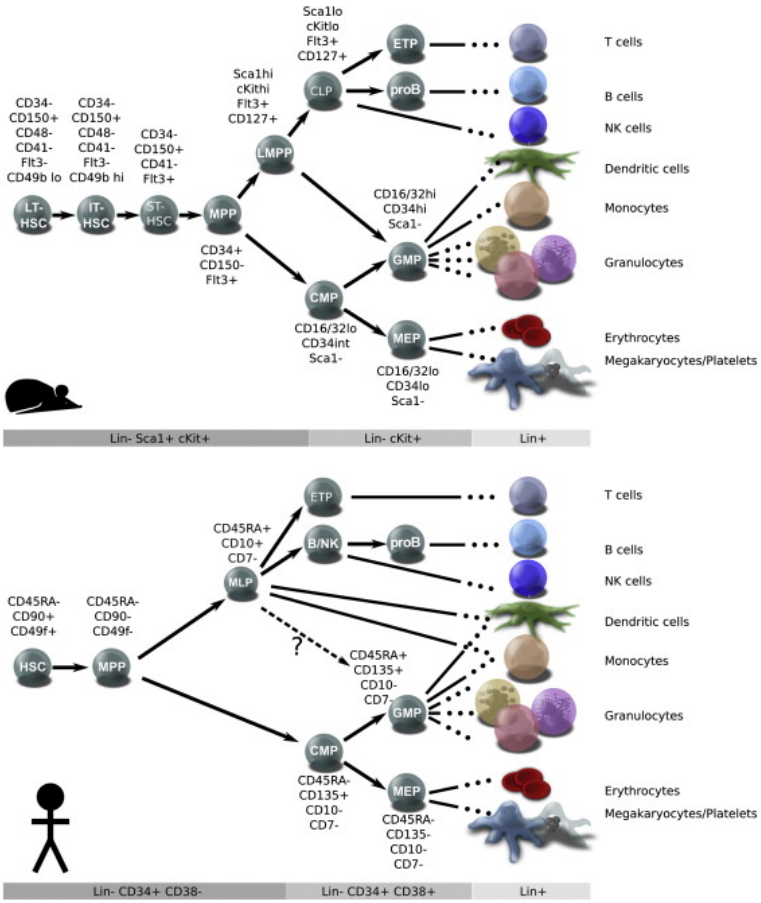


Figure 6: *Developmental model for hematopoietic stem cell differentiation in mouse and human.*

HSC: hematopoietic stem cell, LT: long-term, IT: intermediate-term, ST: short-term; MPP: multipotent progenitor population; LMPP: lympho-myeloid progenitor population; CLP: common lymphoid progenitor; CMP: common myeloid progenitor; ETP: early thymic progenitor; GMP: granulocyte-monocyte precursor; MEP: megakaryocyte-erythrocyte progenitor; MLP: myelo-lymphoid progenitor (Figure adapted from Doulatov et al. (70))

However, the surface phenotype of HSC, especially of cells generated or manipulated *in vitro* does not always correlate with their functional capacities. It is therefore required to validate the phenotype by *in vitro* or *in vivo* functional assays for stem cell function.

Multipotency can be tested *in vitro* using colony forming unit (CFU) assays. For this assay, cells are cultured in semisolid medium containing a mixture of hematopoietic cytokines. As the medium is semisolid and migration of cells is limited, the progeny of one single cell can be assessed. The progeny is usually scored for red blood cells, megakaryocytes and myelomonocytic cells. However it has to be noted that lymphoid potential is not addressed using this CFU assay. Alternatively, presumptive HSC can be tested in a differentiation assay where a single cell of the population of interest is deposited per well. By addition of instructing cytokines and feeder cells, cells can be differentiated towards erythroid, megakaryocytic, myeloid and lymphoid cells. The phenotype of this progeny can then be assessed using flow cytometry.

The capacity to engraft and to self-renew can only be addressed using an *in vivo* assay: the SCID repopulating cell assay (SRC) (72). Here, sublethally irradiated immune deficient mice are injected with a human hematopoietic progenitor cell suspension. After 6-8 weeks, engraftment is scored by assessing human/mouse chimerism in the blood. Self-renewal is assessed by secondary or tertiary transplantation of bone marrow from the engrafted animals (73).

3.8 Hematopoietic differentiation of human pluripotent stem cells

Since the initial derivation of hESC several protocols have been described to induce their hematopoietic differentiation (74-80). Differentiation towards several hematopoietic lineages has been described from PSC. NK cells (81), T cells (82), megakaryocytes (83), erythrocytes (84), monocytes and granulocytes (85) have all been generated through *in vitro* differentiation of hESC and hIPSC .

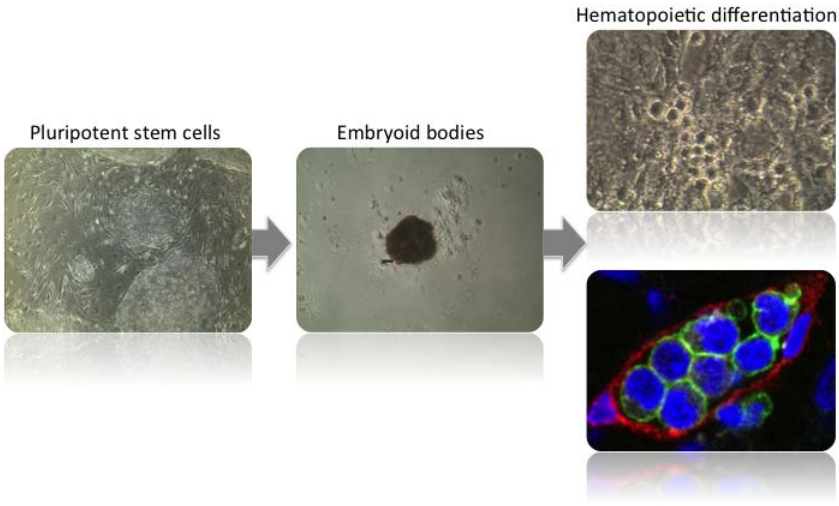


Figure 7: *In vitro hematopoietic differentiation of human embryonic stem cells.*

Pluripotent stem cell colonies are dissociated and are re-aggregated to form embryoid bodies. After 7-10 days, clear "hematopoietic zones" are visible. Here endothelial cells ($CD34^+$, red) are lining hematopoietic cells ($CD43^+$, green).

hESC differentiation protocols are mainly based on two methods: stroma based differentiation methods (74, 75, 86) and embryoid body (EB) differentiation methods (76-78). For the first method, undifferentiated PSC colonies are fragmented and transferred onto a stromal cell layer, known to support hematopoietic differentiation. Examples of such cell lines are the OP9 cell line (derived from the bone marrow of op/op mice) (87) and the S17 cell line (generated through long-term culture of bone marrow from BALB/cAN mice) (88). For the EB method small fragments of undifferentiated hESC, or a suspension of single cells, are allowed to re-aggregate in a small clump and are subsequently allowed to differentiate.

In standard differentiation protocols, feeder cells of murine origin are used or media containing components of animal origin are used for culture. Most of these protocols generate blood cells with relatively low efficiency with 1-10% $CD45^+$ cells. If PSC-derived hematopoietic cells are to be used in a clinical setting, so called xeno-free protocols are needed. Xeno-free protocols are devoid of any component of animal origin and make use of recombinant growth factors. To date, few of these methods have been described (79, 89).

In vitro hematopoietic hESC differentiation seems to closely mimic the steps through which *in vivo* hematopoiesis occurs (Figure 5) (64). Upon differentiation of the hESC, these go through a mesodermal stage, before they generate hemangioblast cells, which have the potential to form endothelial and hematopoietic cells. The subsequent stages during *in vitro* hematopoiesis can be easily distinguished by two markers: sialomucin gp105-120 (CD34) and leukasialin (CD43) (Figure 8) (90). CD34 marks endothelial cells and hematopoietic progenitor cells, while CD43 is the earliest pan hematopoietic marker found in hESC differentiation cultures.

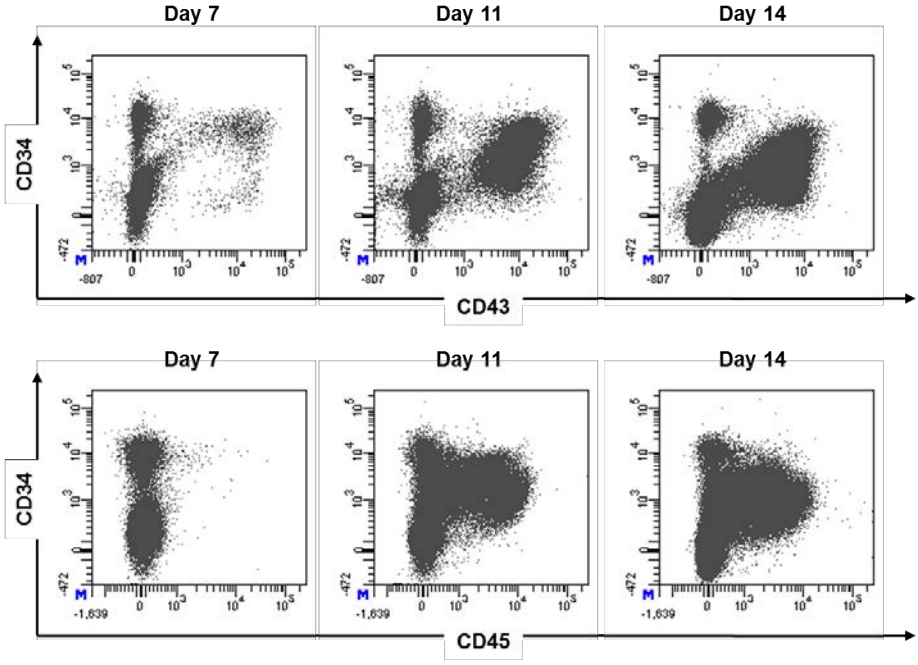


Figure 8: *Kinetics of in vitro hESC hematopoiesis.*

Representative dot plots are shown for EB's analyzed after 7, 11 and 14 days of culture. Cells were stained with the endothelial cell and progenitor cell marker CD34, the early pan hematopoietic marker CD43 and the late pan hematopoietic marker CD45.

After 5-6 days of culture, endothelial cells can be detected in these cultures. These are marked by strong expression of CD34 and lack of CD43 (Figure 8). After 7-8 days, the first erythroid and megakaryocytic cells can be detected and these are negative for CD34 but positive for CD43. Subsequently definitive type hematopoietic progenitors can be found which increase in numbers from day 10 onwards and are marked by expression of both CD34 and CD43. Later on in culture, blood cells start to express the late pan hematopoietic marker CD45. The structures generated in

these cultures show high resemblance to the blood islands found in the YS, where hematopoietic cells are lined by endothelial cells. The general concept that hemogenic endothelial cells give rise to hematopoietic cells, also holds true for *in vitro* hematopoiesis of hESC (91, 92). Culture of purified $CD34^{+}CD43^{-}CD45^{-}$ endothelial cells readily generate hematopoietic cells, however the frequencies of these cells remain low.

Several reports have claimed generation of hematopoietic progenitor cells capable of engraftment from hESC *in vitro* (93, 94). However, clear differences can be found between these hESC-derived multipotent progenitor cells and somatic HSC. While CFU progenitor frequencies have been described to be similar between somatic HSC and hESC-derived hematopoietic precursor cells (HPC), the hESC-derived HPC show a skewed CFU potential towards granulocytic cells compared with the potential of somatic HSC (93). After intrafemoral injection of hESC-derived HPC, engraftment remains low in the bone marrow compared with somatic HSC (10-100 fold lower). The bone marrow was found to contain ~0.1-1% human blood cells upon engraftment of hESC derived HPC (94). In these reports no evidence is provided for multilineage repopulation (93, 94). In a more recent report, hESC were differentiated towards hematopoietic cells on different stromal cell lines derived from murine urogenital ridges (UG), AGM and FL (75). This led to engraftment of hESC-derived HPC in the contralateral femur after intrafemoral injection. Human cells were found to constitute around 2% of bone marrow cells and peripheral blood contained around 16% human blood cells. Again, ample evidence is provided for multilineage repopulation, and the authors themselves claim that they cannot rule out the presence of long lived myeloid cells.

Taken together, these reports do show engraftment of hESC-derived hematopoietic cells, but with low efficiency. Cells which can be serially transplanted and are capable of generating all blood cell lineages have not been unequivocally described. Moreover, hESC-derived HPC all seem to show the tendency to differentiate towards myeloid progenitors upon injection. Therefore, to date no convincing evidence has been provided for the *in vitro* generation of cells with HSC properties.

Recent reports have described the generation of lympho-myeloid precursors (LMP) and erythro-myeloid precursors (EMP) cells during early definitive YS hematopoiesis in the murine system. In addition, striking similarities between the timing of

hematopoiesis and the potential of the cells generated during *in vitro* hESC differentiation and *in vivo* human hematopoiesis have been described (64). It might thus not be illogical to hypothesize that *in vitro* hESC hematopoietic differentiation might mimic YS definitive hematopoiesis, without the formation of HSC.

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5. Research focus of part I

PSC have the capacity to generate every cell type of the body. Not soon after the derivation of embryonic stem cells, protocols were published to differentiate these cells into hematopoietic cells. As almost every hematopoietic cell type has been generated starting from pluripotent stem cells, a common misconception is that a HSC must lie at the basis of these different cell types.

This presumptive generation of HSC, led to several publications claiming successful engraftment of PSC derived HSC in immune deficient animals. Together with the description of iPSC from patient derived somatic cells, this has raised high hopes in generating patient specific transplantable HSC for the clinic.

Study of hematopoiesis in the murine system is uncovering the hematopoietic system to be even more complex than previously appreciated. Before a cell with HSC properties is formed in the embryo, a whole array of hematopoietic precursors is generated in the YS and the AGM/splanchnopleura. These precursors have limited or no engraftment capacity. In the YS, which is the extra-embryonic part of the developing fetus, progenitors are formed which are able to generate erythroid, myeloid and lymphoid cells. The fact that all hematopoietic cell lineages are found in culture, therefore, in no way implies the presence of a HSC.

We hypothesized that during *in vitro* PSC derived hematopoiesis one of the former two types of hematopoiesis could occur. Either a cell with HSC characteristics is formed (AGM-like hematopoiesis), or more restricted progenitors are formed (YS-like hematopoiesis). Previous studies addressed this by testing the engraftment capacity of the cells generated, albeit with limited success. In the study described here, we used an alternative *in vitro* strategy. We based our hypothesis on the essential role which has been described for the transcription factor MYB in the function of HSC. MYB has been described to be expressed at high levels in HSC, and expression levels decrease during differentiation towards lineage progenitors. On the other hand, YS-based hematopoiesis has been described to be independent of MYB. We thus put forward that expression of MYB during the formation of hematopoietic progenitor cells would allow us to discriminate between the emergence of a MYB^+ HSC or a MYB^- YS-like precursor.

To this end we have generated a MYB-eGFP human embryonic stem cell line and assessed the latter hypothesis during *in vitro* hESC differentiation.

The results of this study call for a critical re-assessment of previously published papers, as we found no cells with HSC characteristics, expressing high levels of MYB. Rather, hematopoiesis in these cultures seems to closely resemble YS based hematopoiesis, with the formation of tissue macrophages. The only MYB signal that could be detected, was during granulocytic commitment of these progenitors.

We believe that use of a reporter cell line as the one we described, and adaptation of culture protocols, will aid the field in the search for factors which induce the generation of HSC from pluripotent stem cells.

Given the recent insights in hematopoietic development both *in vitro* and *in vivo*, and the data generated in our study, we discuss the relevance of these findings for gene therapy of hematological diseases.

6. Publications

6.1 In vitro human embryonic stem cell hematopoiesis mimics MYB-independent yolk sac hematopoiesis.

Stijn Vanhee, Katrien De Mulder, Yasmine Van Caeneghem, Greet Verstichel, Nadine Van Roy, Björn Menten, Imke Velghe, Jan Philippé, Dominique De Bleser, Bart N Lambrecht, Tom Taghon, Georges Leclercq, Tessa Kerre and Bart Vandekerckhove

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Q1- Ranked 5/68 (Hematology)

***In vitro* human embryonic stem cell hematopoiesis mimics MYB-independent yolk sac hematopoiesis.**

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Abstract

Although hematopoietic precursor activity can be generated *in vitro* from human embryonic stem cells, there is no solid evidence for the appearance of multipotent, self-renewing and transplantable hematopoietic stem cells. This could be due to short half-life of hematopoietic stem cells in culture or, alternatively, human embryonic stem cell-initiated hematopoiesis may be hematopoietic stem cell-independent similar to yolk sac hematopoiesis, generating multipotent progenitors with limited expansion capacity. Since Myb was reported to be an excellent marker for hematopoietic stem cell-dependent hematopoiesis, we generated a MYB-eGFP reporter human embryonic stem cell line to study formation of hematopoietic progenitor cells *in vitro*. We found $CD34^+$ hemogenic endothelial cells rounding up and developing into $CD43^+$ hematopoietic cells without expression of MYB-eGFP. MYB-$eGFP^+$ cells appeared relatively late in embryoid body cultures as $CD34^+CD43^+CD45^{-/lo}$ cells . These MYB-$eGFP^+$ cells were CD33 positive, proliferated in IL-3 containing media and hematopoietic differentiation was restricted to the granulocytic lineage. In agreement with data obtained on murine $Myb^{-/-}$ embryonic stem cells, bright eGFP expression was observed in a subpopulation of cells, during directed myeloid differentiation, which again belonged to the granulocytic lineage. In contrast, $CD14^+$ macrophage cells were consistently $eGFP^-$ and were derived from $eGFP^-$ precursors only. In summary, no evidence was obtained for *in vitro* generation of MYB^+ hematopoietic stem cells during embryoid body cultures. The observed MYB expression appeared late in culture and was confined to the granulocytic lineage.

Introduction

During embryogenesis, hematopoietic development is spatiotemporally organized in different waves. During the first, so-called primitive wave of hematopoiesis, nucleated erythrocytes and macrophage-like cells are generated in the yolk sac (YS). Subsequent waves generate multipotent progenitor cells, first in the YS and, finally, in the aorta-gonado-mesonephros region (AGM) where hematopoietic stem cells (HSCs) are generated (1).

Members of the MYB transcription factor family are important transcriptional regulators throughout embryonic development. One of these three family members, MYB, is differentially expressed during embryonic hematopoietic development (2). While Myb transcripts have been detected at low levels during early waves of hematopoiesis, this process is not MYB-dependent. In contrast, HSC-dependent hematopoiesis appears to strictly rely on MYB as loss of function mutants of MYB lead to embryonic lethality due to failure of fetal liver hematopoiesis (3, 4). In the zebrafish, transgenic animals expressing eGFP under control of myb regulatory elements have been used for visualization and analysis of HSC generation from aortic blood forming hemogenic endothelium (5).

In postnatal life, long term repopulating hematopoietic stem cells (LT-HSC) were found to express the highest levels of Myb and levels decrease progressively in short term repopulating HSC (ST-HSC) and in the multipotent progenitor population (MPP)(6, 7).

MYB-independent hematopoiesis consists largely of short-lived precursors and mature blood cells. However, the mononuclear phagocyte system (MPS), consisting of brain microglial cells, hepatic Kupffer cells and skin Langerhans cells, seems to be derived from MYB-independent hematopoietic progenitor cells (HPC) rather than bone marrow-derived MYB-dependent HSC. Recently, Schulz and colleagues have shown that two parallel pathways of macrophage differentiation can be distinguished by their inherent dependence on MYB (8). MYB-independent cells originate in the yolk sac around E7.5-E8.5, possibly from an erythro-myeloid restricted precursor (EMP), while MYB-dependent macrophages originate from bone marrow HSC.

Gene therapeutic strategies for inherited immune deficiencies or other genetic diseases of the blood rely on the assumption that HSC can be generated *in vitro* from pluripotent stem cells(9). In these approaches, patient-specific induced pluripotent stem cells are generated and the defective gene is corrected by homologous recombination subsequent to targeting the gene defect by zinc-finger nucleases (ZFN) or TALENs. Once the genetic defect is corrected, “cured” HSC are generated by an appropriate *in vitro* differentiation protocol before infusion. However, current protocols have failed to convincingly demonstrate the generation of HSC in pluripotent stem cell differentiation cultures.

To investigate whether during *in vitro* human embryonic stem cell (hESC) derived hematopoiesis HSC are formed or rather, hematopoiesis from hESC depends on the emergence of a myb independent EMP-like cell, we generated a MYB reporter line using random integration of a bacterial artificial chromosome (BAC) reporter construct in which eGFP expression is under control of the MYB regulatory DNA sequences.

Methods

Cell lines, culture of cell lines and isolation of primary cells

All experiments were approved by the Medical Ethical Committee of Ghent University Hospital (Belgium). The WA01 (National Institutes of Health code: WA01) human embryonic stem cell (hESC) line was used in all experiments. Further methods describing used cells and culture of cells can be found in supplemental materials.

Hematopoietic differentiation of hESC in spin embryoid bodies (EB)

To differentiate hESC into hematopoietic cells, the protocol from Ng et al. was used with minor modifications(10). In brief, 5x103 single cell-adapted hESC were spun at 480 g into each well of a 96-well low attachment plates and subsequently cultured in APEL medium containing 10 µM Rock inhibitor Y-27632 (Selleckchem, Houston, TX, USA), 40 ng/ml SCF (Peprotech, Rocky Hill, NJ, USA), 2 ng/ml BMP4 (R&D, Minneapolis, MN, USA) and 20 ng/ml VEGF165 (Peprotech), further referred to as "EB mix". After 4 days, spin EB were transferred on an OP9 cell layer and further cultured in EB mix for a total of 7-14 days. Half of the medium was changed on day 7, with APEL medium containing EB mix cytokines, unless a different combination of cytokines is specified. These cytokines were added at following concentrations: 50 ng/ml IL-3 (R&D), 50 ng/ml Flt3-L (R&D), 10 ng/ml TPO (Peprotech) and/or 50 ng/ml IL-6 (R&D).

For myeloid differentiation, spin EBs were dissociated at day 11 and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech), 10 ng/ml IL3 (R&D) and 20 ng/ml IL-6 (R&D). For erythro-megakaryocytic differentiation, spin EBs were dissociated and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech) and 50 ng/ml EPO (eBioscience, San Diego, CA, USA).

Flow cytometry and cell sorting

Flow cytometric analysis was performed on a LSR II system (BD biosciences, San Jose, CA, USA). Cell sorting was performed with a FACS ARIA IIIU system (BD Biosciences). A list of antibodies used can be found in supplemental materials.

Real time RT-PCR

Cells were lysed and cDNA was synthesized using the SYBR power cells-to-Ct system (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Primer sequences can be found in supplemental Table S1. PCR reagents and SYBR GreenI master were obtained from Roche (Roche, Penzberg, Germany) and used according to the manufacturer's instructions. The reactions were run on a lightcycler480, 384well system (Roche).

Statistical analysis

All statistical analyses were performed using SPSS V22.0 (IBM, New York, USA). Significance was assessed using Mann-Whitney U statistical analysis with significance level set at $p \leq 0.05$.

Additional materials and methods are provided in supplemental materials and methods file.

Results

Generation of MYB-eGFP transgenic reporter hESC.

To study MYB expression during hematopoietic differentiation, we generated a MYB-eGFP reporter hESC line using random insertion bacterial artificial chromosome (BAC) transgenesis (supplemental figure 1).

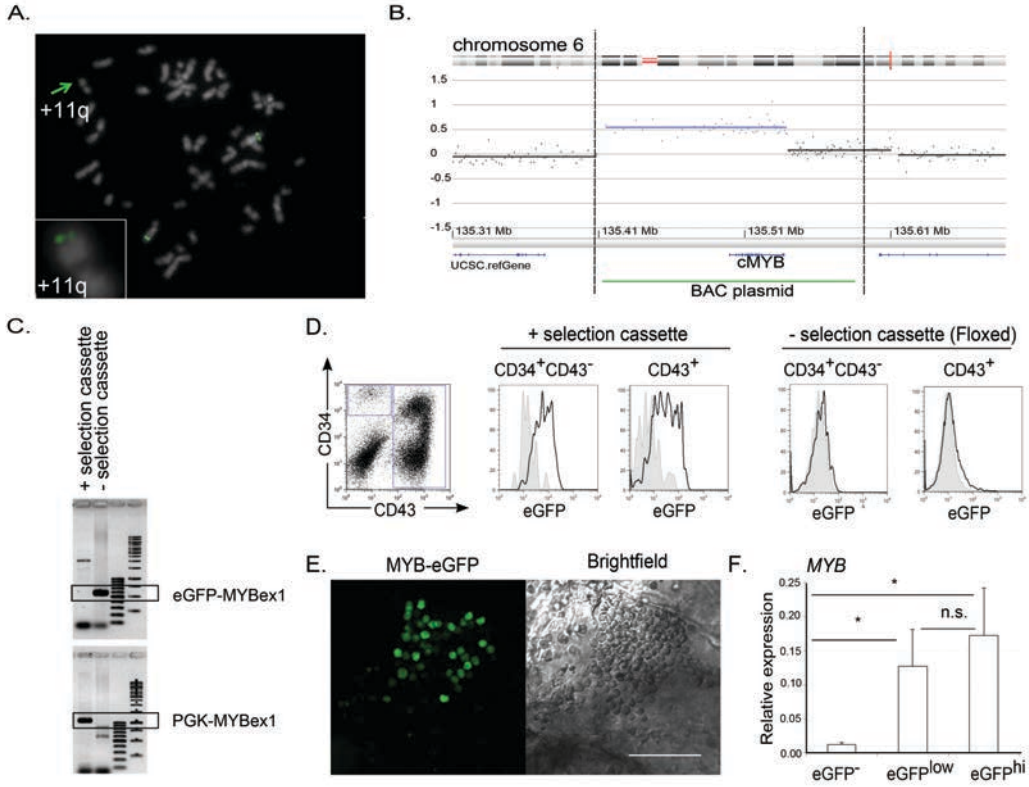

Figure 1: Functional validation of MYB-eGFP reporter hESC.

A) MYB FISH analysis (green) on metaphase nuclei (DAPI stain in grey); inset shows chromosome 11 only; B) ArrayCGH showing amplification of the genomic region within the BAC plasmid. A relevant portion of chromosome 6 is shown; c) Validation of selection cassette removal, as assessed by PCR ranging from eGFP to the first exon of MYB (upper gel) or ranging from pPGK to the first exon of MYB (lower gel); D) MYB-eGFP reporter hESCs differentiated towards the hematopoietic lineage were analyzed for eGFP expression. $CD34^+CD43^-$ endothelial and $CD43^+$ hematopoietic populations are depicted. Both floxed and non-floxed cell lines are shown; E) Confocal fluorescence microscopy of a day 20 EB culture in EB mix showing bright eGFP positive round cells, scale bare measures 100µM; F) MYB qPCR analysis of eGFP sorted hematopoietic cells from EB differentiation culture. Expression is shown relative to the mean of GAPDH and YWHAZ expression. Error bars indicate standard deviation (SD) of the mean (n=4).

After nucleofection of hESC and neomycin selection, neomycin-resistant hESC colonies were obtained. In figure 1, data are shown of clone 5, which gave highest eGFP expression and was used in all experiments. Besides the endogenous MYB loci on chromosome 6q, a clear hybridization signal on chromosome 11q was detected on FISH analysis, confirming successful integration of the BAC reporter vector (Figure 1 A). High-resolution arrayCGH shows integration of the BAC plasmid from +84.6 kbp 5' of the MYB start codon to -39.3 kbp 3' of the start codon. This region encompasses the complete MYB gene and upstream regulatory elements (Figure 1 B).

Hematopoietic differentiation of the MYB-eGFP cell line, before removal of the selection cassette, showed eGFP expression by $CD43^+$ hematopoietic cells as well as by $CD34^+CD43^-$ endothelial cells (Figure 1 D). As no MYB transcripts could be found by qPCR analysis in the $CD34^+CD43^-$ population (supplemental figure 2), aberrant expression due to interference of the pGK promotor was hypothesized. Therefore, the pGK promotor-driven selection cassette was removed through transient transfection with a Cre recombinase-encoding plasmid (Figure 1 C). After removal of the selection cassette, no eGFP expression was observed in non-hematopoietic cells (Figure1 D). Although the expression of eGFP found at this point in time was relatively weak, prolonged culture, clearly showed strong $eGFP^+$ cells as determined by confocal microscopy analysis of d20 EB differentiation cultures (Figure 1 E).

Fidelity of the random integration reporter cell line was validated by qPCR analysis for MYB expression. $eGFP^-$, $eGFP^{low}$ and $eGFP^{hi}$ cells were sorted from d14 hematopoietic differentiation cultures in the presence of SCF, TPO, IL-3 and IL-6 and MYB mRNA levels were shown to correlate with eGFP protein expression in the sorted populations (Figure1 F and supplemental figure 2). Note that the eGFP- cells are not completely negative for MYB mRNA. This indicates that the reporter line is not as sensitive as RT-qPCR for MYB expression. However, this does not preclude the detection of HSC as these cells are supposed to express high levels of *Myb*(6).

Endothelium-derived early hematopoietic precursors are MYB-eGFP negative.

Spin EB cultures were set up and screened daily for the appearance of hematopoietic $eGFP^+$ cells. Expression of CD43, the earliest marker for hematopoietic cells, was first observed in our cultures at day 7. Few $eGFP^+$ cells became apparent from day 11 onwards, forming a clear population on day 14. As shown in Figure2 A-B, these $eGFP^+$ cells were contained within the $CD34^+CD43^+$ population, which has been reported to contain progenitors of multiple hematopoietic lineages. On the other hand the $CD34^+CD43^-$ endothelial and $CD34^+CD43^{lo}$ emerging HPC populations were consistently negative for eGFP expression (Figure 2 B). As expression was also found highest in the $CD34^+CD43^+$ population within the $CD45^{low}$ population (supplemental figure 2), we hypothesized this population to have the highest possibility of containing MYB^+ multipotent progenitors.

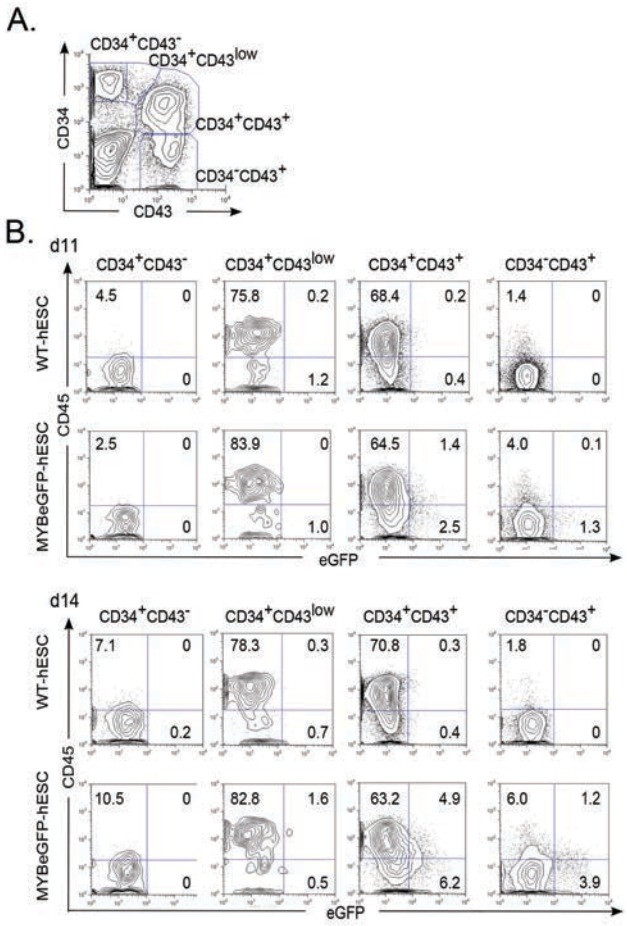

Figure 2: MYB-eGFP+ HPC are generated relatively late in EB cultures.

A) Gating strategy for assessment of eGFP expression, a representative plot of EB day 11 culture is shown; B) Flow cytometric analysis of day 11 and day 14 embryoid body cultures in EB mix. Wild type hESC are presented as control for eGFP signal in all populations. Percentages of cells in each quadrant are shown.

To assess whether the eGFP$^+$CD43$^+$ hematopoietic cells were emerging directly from hemogenic endothelium, day 11 CD34$^+$CD43$^-$ endothelial cells were sorted and replated on OP9 stromal cells in medium containing SCF, BMP4 and VEGF. After 6 days of culture a distinct CD45$^+$ population was apparent, however no eGFP$^+$ cells were seen at this timepoint. After 11 days of culture, eGFP$^+$ cells became apparent (supplemental figure 3 A). As this assay might miss the emergence of transient eGFP$^+$ cells directly from hemogenic endothelium, we analyzed using a similar set-up, the emergence of hematopoietic cells from endothelium by live confocal imaging. During live confocal imaging, adherent eGFP$^-$CD34$^+$CD43$^-$ endothelial cells were

found to round up and form cells with hematopoietic appearance. These cells subsequently upregulated CD43 and proliferated extensively over the course of 72 hrs. We could not detect eGFP expression during the whole process of blood cell generation and subsequent proliferation, suggesting MYB-independent generation of HPC (Figure 3 and Supplemental Movie S1). Similar experiments initiated with sorted $eGFP^{-}CD34^{+}CD43^{-}$ endothelial cells derived from earlier (day 7) or later time points (day 14) of EB cultures were much less potent in the generation of hematopoietic cells and generated only few blood cells.

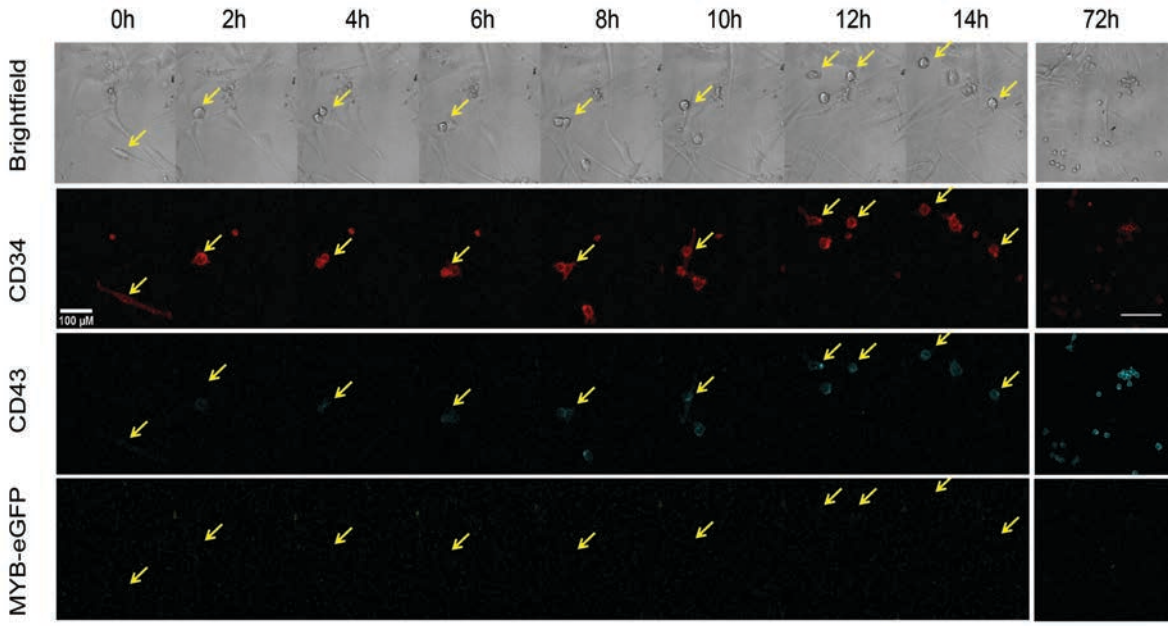

Figure 3: Hematopoietic cells are generated directly from hemogenic endothelium, without and eGFP+ intermediate.

Time-lapse confocal analysis of replated day 11 hemogenic endothelium. A single frame is depicted every two hours starting from the defined starting point. Cropped images are shown for every channel. Arrows depict a single endothelial cell at the starting point of analysis and the progeny thereof at later points in time. Scale bar measures 100 µM; At 72h, the cells have rapidly proliferated and generated two clusters of 8 cells. These cells have downregulated CD34 and upregulated CD43. Scale bar measures 100µM.

To verify whether other or additional growth factors were required for the generation and/or expansion of the $eGFP^{+}$ $CD34^{+}CD43^{+}CD45^{-/lo}$ population, EB-derived hematopoietic cells were cultured in different cytokine combinations of SCF, TPO,

Flt3L, IL-3 and IL-6. It was found that eGFP+CD34$^+$CD43$^+$CD45$^{-/lo}$ cells expanded most efficiently in conditions containing IL-3. As shown in Figure 4A, the mean of absolute numbers of eGFP$^+$CD34$^+$CD43$^+$CD45$^{-/lo}$ per EB was about six fold higher than those in conditions without IL-3. This suggests that the eGFP$^+$CD34$^+$CD43$^+$CD45$^{-/lo}$ cells are responsive to IL-3, as has been reported previously (11).

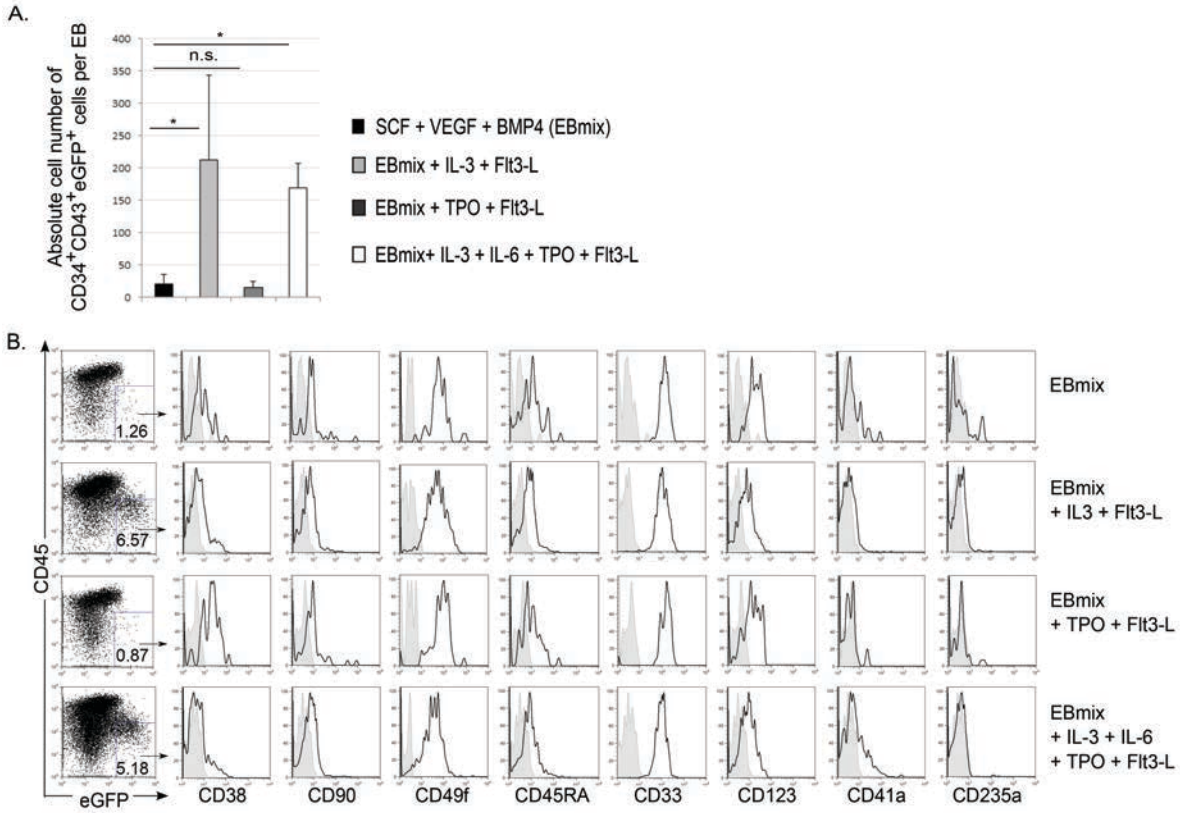

Figure 4: Phenotype of MYB-eGFP$^+$ HPC.

A) Absolute cell number of eGFP$^+$ cells within the CD34$^+$CD43$^+$ population of day 14 embryoid body cultures. Error bars indicate standard deviation (SD) of the mean (n=3); B) Flow cytometric analysis of day 14 MYB-eGFP EB, cultured in the presence of different cytokine mixes as indicated. Dot plots are gated on CD34$^+$CD43$^+$. Cells within the eGFP$^+$ gate are depicted in the histograms. Shaded histograms show isotype control stained samples for the indicated marker. Representative plots of 3 independent experiments are shown;

Characterization of eGFP negative and eGFP positive subsets.

To further define the $eGFP^+$ cells generated in these cultures, we analyzed these cells for expression of the stem cell phenotype $CD38^-$, $CD90^+$, $CD49f^+$ and $CD45RA^-$ as previously described by Notta et al., the myeloid lineage markers CD33 and CD123, and erythro-megakaryocytic lineage markers CD41a and CD235a. As shown in figure 4B, $eGFP^+CD34^+CD43^+CD45^{-/lo}$ cells expressed low levels of CD45RA, were negative for CD38 and CD90 and expressed CD49f. This $CD34^+CD45RA^-CD38^-CD49f^+$ phenotype was present in all conditions including the conditions containing IL-3, and is similar to the long term engraftable HSC described by Notta *et al*(12) (Figure 4B). However, the $eGFP^+$ cells in our cultures also showed clear expression of CD33 and CD123, while erythroid lineage markers were negative, suggesting myeloid lineage commitment of these cells. The phenotype was very similar independent of the cytokines added, except for the condition in EBmix + TPO + Flt3-L, where CD38 was upregulated. CD235 and CD41a were consistently absent on these populations, arguing against erythroid commitment of $eGFP^+CD34^+CD43^+CD45^{-/lo}$ cells. The $eGFP^-CD34^+CD43^+CD45^-$ cells, on the other hand, contained mainly erythroid committed cells, while the $CD45^+$ population, consisted of cells expressing myeloid lineage markers (data not shown). The $eGFP^+$ cells derived from $eGFP^-$ hemogenic endothelium, also phenotyped as $CD45^{low}CD33^+CD14^-$ cells, consistent with the data obtained on bulk cultures (supplemental figure 3 B)

We assessed myeloid, erythroid and megakaryocytic differentiation capacity of the different populations obtained in the cultures with various growth factor mixes to assess multipotency. Results were qualitatively similar for all growth factor conditions. In figure 5 A, the results are shown of sorted cell populations from d14 spin EB cultures expanded with EBmix plus IL-3 and Flt3-L, and subsequently assayed under conditions optimal for either myeloid, erythroid or megakaryocytic differentiation. The $eGFP^-CD34^+CD43^+CD235a/CD41a^-CD45^-$ population clearly contained progenitors giving rise to myeloid, erythroid and megakaryocytic cells, while the $eGFP^+CD34^+CD43^+CD235a/CD41a^-CD45^{-/lo}$ population was shown to give rise to granulocytic lineage myeloid cells only, with complete absence of monocytic, erythroid and megakaryocytic precursor potential. On the other hand, $eGFP^-$

$CD34^+CD43^+CD235a/CD41a^+$ cells gave rise mainly to erythroid and megakaryocytic cells, although some myeloid precursor activity was also observed. $eGFP^-CD34^+CD43^+CD235a/CD41a^-CD45^+$ cells were able to give rise to $CD11b^+CD14^-$ granulocytic lineage cells, $CD11b^+CD14^+$ macrophage lineage cells, $CD71^+CD235^+$ erythroid and $CD41^+$ megakaryocytic cells (Figure 5 A).

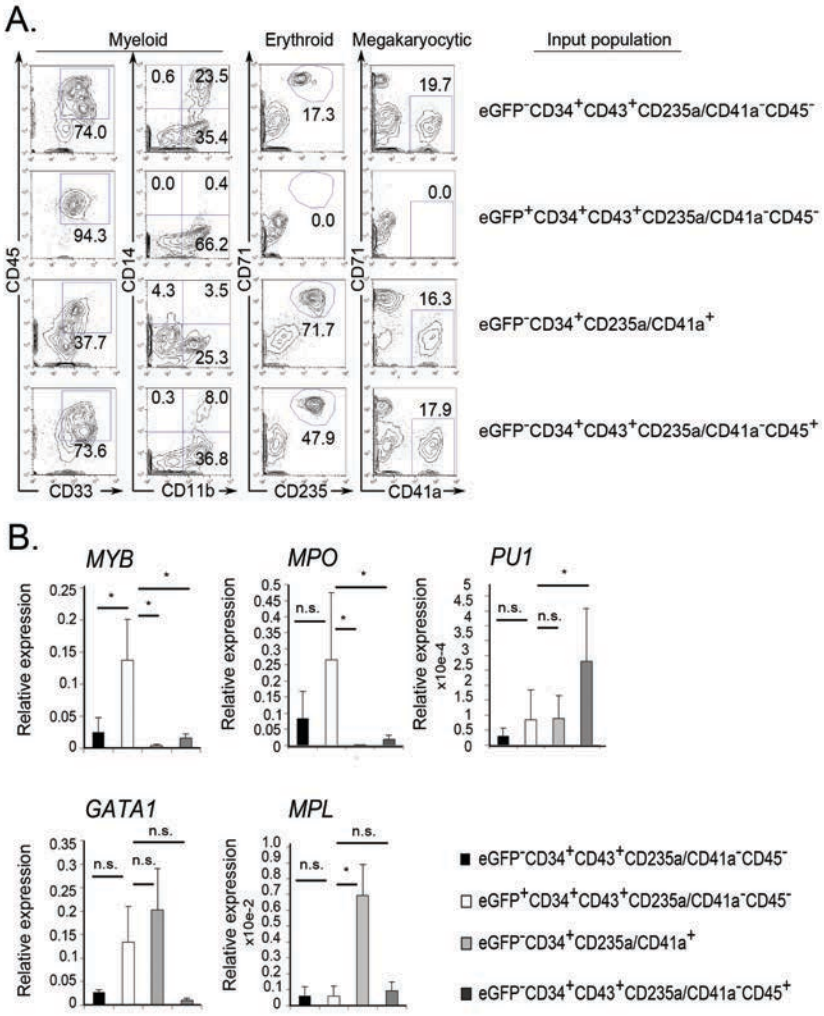

Figure5: MYB-eGFP⁺ HPC show commitment towards the granulocytic lineage.

A) Flow cytometric analysis of differentiation cultures towards granulocytic, monocytic, megakaryocytic and erythroid cells. Day 14 MYB-eGFP hESC-derived precursors with indicated phenotypes were isolated from IL-3 and Flt3-L expanded EB; representative plots of at least 3 independent experiments are shown; B) qPCR analysis for lineage commitment genes on indicated populations, isolated from day 14 MYB-eGFP hESC EB expanded in presence in IL-3 and Flt3-L. Expression is shown relative to the expression of GAPDH. Error bars indicate standard deviation (SD) of the mean (n=3).

To assess expression of genes associated or determining commitment towards the different lineages, RT-qPCR was performed for MPO, GATA1, PU.1 and MPL on the same sorted populations as in Figure 5 A. MYB was assessed as a control and showed high expression in the $eGFP^+$ population as expected (Figure 5 B). In line with the multipotent precursor capacity, the $eGFP^-CD34^+CD43^+CD45^-$ cells expressed only low levels of the various genes analyzed. In contrast, the $eGFP^-CD34^+CD43^+CD45^+$ cells had significantly higher levels of PU.1 suggesting that together with the acquisition of CD45, the cells become committed to the macrophage lineage. As expected, the $eGFP^-CD34^+CD235a/CD41a^+$ express high levels of GATA1 and MPL, emphasizing their erythro-megakaryocytic commitment. In contrast, the $eGFP^+CD34^+CD43^+CD45^{-/lo}$ cells show high expression of MPO and low levels of PU1 suggestive for granulocytic lineage commitment. GATA1 expression was also detected in the $eGFP^+CD34^+CD43^+CD45^{-/lo}$ population, in agreement with granulocytic lineage commitment

To study the generation of the $eGFP^+$ precursor at the clonal level, CFU assays and single cell sorting followed by liquid culture were performed on d11 and d14 EB cultures (figure 6). The day11 $eGFP^-CD34^+CD43^+$ cell population gave rise to CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E and CFU-E. These colonies were analyzed microscopically for eGFP expression and it was observed that the CFU-GM, CFU-G and CFU-GEMM contained eGFP positive cells whereas the CFU-M, CFU-E and BFU-E were consistently negative (figure 6 A). Similarly, using single cell culture and flow cytometric analysis, we found that d11 and d14 $eGFP^-CD34^+CD43^+$ progenitors consisted of GEMM, GM, G, M, E precursors. A marked skewing towards the myeloid lineage was observed on d14 of culture. In agreement with the CFU assays, $eGFP^+$ cell containing wells were confined to the wells containing G, GM and GEMM precursor cells, whereas the wells lacking eGFP expression contained E and M precursors (figure 6 B and 6 C). These data clearly show, that the MYB-$eGFP^+$ precursors is a more committed myeloid precursor derived from a multipotent MYB-$eGFP^-$ hematopoietic precursor cell.

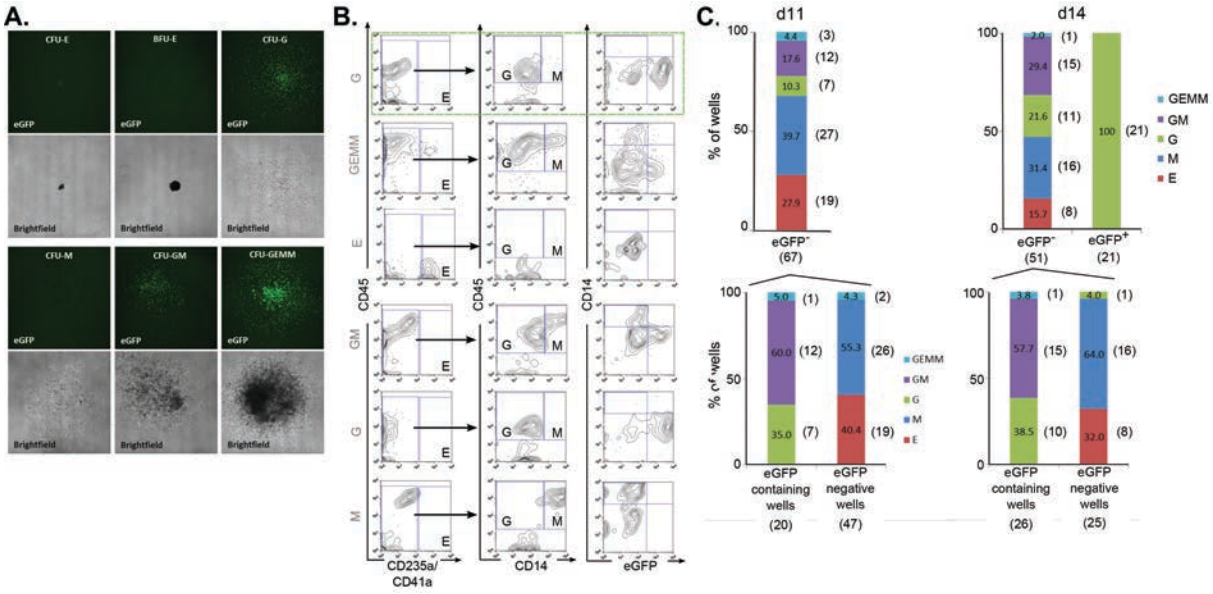

Figure 6: Clonal progeny analysis of CD34$^+$CD43$^+$ progenitors.

A) Representative microscopic images of eGFP$^-$CD34$^+$CD43$^+$ d11 EB derived progenitor CFU assay, analyzed after 14 days of liquid culture. A representative image for each colony type is depicted. Both eGFP and brightfield channels are shown; B) Representative analysis of single cell (clonal) cultured CD34$^+$CD43$^+$ EB derived progenitors, analyzed after 7 days of culture. CD235a/CD41a$^-$ cells are depicted in the center and right panels. Scoring gates are indicated with E (erythroid/megakaryocytic), M (monocytic) and G (granulocytic). Combination of these gates led us to determine colony type as G, M, E, GM or GEMM as indicated for the representative plots. Representative progeny of eGFP$^+$CD34$^+$CD43$^+$ EB derived progenitors are depicted in the dotted box; C) Frequency of progenitor types within CD34$^+$CD43$^+$ EB derived progenitors at the indicated time points. Absolute numbers of colonies are indicated between brackets. The progeny of eGFP$^-$CD34$^+$CD43$^+$ are depicted in the panels below as fractions of wells containing eGFP$^+$ cells or wells lacking eGFP$^+$ cells; all wells were scored as shown in panel B.

While macrophage development is MYB-independent, MYB marks granulocytic lineage differentiation.

As MYB was described to be dispensable for the generation of YS derived macrophages, we studied the role of MYB during myeloid lineage choice. When EB cultures were grown under myeloid differentiation conditions, a homogenous population of CD45$^+$CD33$^+$ was obtained after 4 days of culture (Figure 7 A). To

further define both eGFP$^-$ and eGFP$^+$ populations, we analyzed both populations by flow cytometry, cytospin and qPCR.

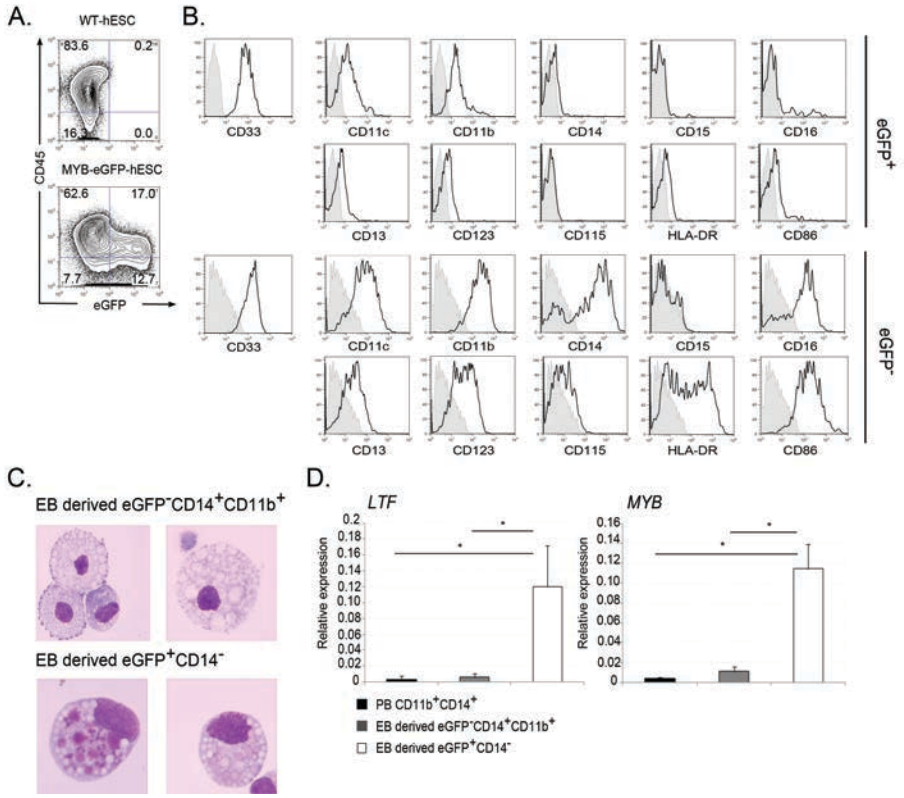

Figure 7: Myeloid cells show strong expression of MYB-eGFP upon granulocytic lineage differentiation.

A) Flow cytometric analysis of myeloid-directed differentiation of day 11 MYB-eGFP hESC embryoid bodies, after 5 days of myeloid culture. Representative plots of at least 3 independent experiments are shown; B) phenotypic analysis of eGFP$^+$ (upper panels) and eGFP- (lower panels) populations in myeloid directed differentiation cultures of day 11 MYB-eGFP embryoid bodies after 5 days of myeloid culture. All cells shown in histograms are gated on CD45. Shaded histograms show control staining for the depicted marker. Representative plots of 3 independent experiments are shown; C) May-Grünwald-Giemsa staining of cytospin samples of myeloid-directed differentiation of day 11 MYB-eGFP hESC embryoid bodies, eGFP$^-$CD14$^+$CD11b$^+$ and eGFP$^+$CD14$^-$ populations are depicted; D) qPCR analysis for lactoferrin (LTF) and MYB expression in indicated populations. Expression is shown relative to the mean of GAPDH and YWHAZ expression. Error bars indicate standard deviation (SD) of the mean (n=2).

The $eGFP^+$ population was found to be $CD45^{lo}$ and positive for CD33, weakly expressing the myeloid markers CD11c, CD11b, CD123 and CD13. The granulocytic marker CD15 was found absent. This phenotype is in line with the surface phenotype of myelocytes, a precursor of the granulocyte lineage. Macrophage markers CD14, CD16, CD115, HLA-DR and CD86 were consistently negative. On the other hand, the $eGFP^-$ population was found to be $CD45^{hi}$, $CD33^+$. These cells were $CD11c^{hi}$, $CD11b^{hi}$, $CD14^+$, $HLA\text{-}DR^+$, $CD86^+$ compatible with a activated macrophage phenotype. CD115, the receptor for M-CSF, was found only weakly positive. The cells expressed CD16, which is normally expressed on tissue macrophages (Figure 7 B).

On cytological analysis using May-Grünwald-Giemsa staining, $eGFP^+CD14^-$ cells show a granulocytic cytoplasm compatible with myelocytic granulocyte lineage cells, whereas the $eGFP^-CD14^+$ cells have a morphology compatible with tissue macrophages (Figure 7 C). This was confirmed by qPCR analysis showing these $eGFP^+$ cells to express the granule protein lactoferrin (LTF), eGFP- cells on the other hand were devoid of lactoferrin expression (Figure 7 D). As expected, MYB expression levels were high in $eGFP^+CD14^-$ cells (Figure 7 D).

To analyze whether the generation of these macrophages from hematopoietic precursor cells was MYB-independent, we sorted $eGFP^-CD34^+CD43^+CD45^+$ committed precursor cells, cultured these in myeloid conditions on OP9 stromal cells and analyzed them daily for the expression of eGFP. After 4 days, a population of $CD14^+$ cells was already clearly visible. These cells were negative for eGFP as assessed by flow cytometry (Figure 8 A). Further culture of these cells for up to 11 days, showed clear eGFP positive populations at all analyzed time points. However, at no time point were $eGFP^+CD14^+$ cells observed. After 11 days of culture, virtually all $eGFP^-$ cells were CD14 positive (Figure 8 A).

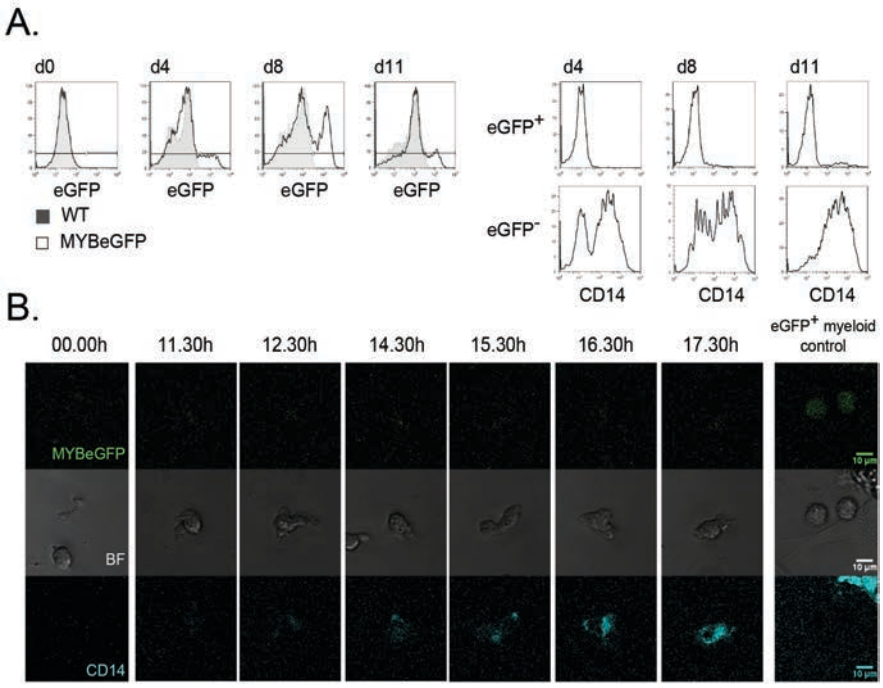

Figure8: Macrophages develop without expression of MYB-eGFP and show characteristics of yolk sac -derived tissue macrophages.

A) Flow cytometric analysis of myeloid-directed differentiation of day 11 MYB-eGFP hESC EB-derived $CD34^+CD43^+CD45^+eGFP^-$ precursors, after 4, 8 and 11 days of myeloid culture; B) Time-lapse confocal analysis of live stained cultures. $CD34^+CD43^+CD45^+eGFP^-CD14^-$ cells were cultured in myeloid differentiation cultures. Samples were live stained with CD14-PE for confocal analysis. $eGFP^+$ myeloid cells are shown as reference. Single channel panels are shown every hour starting from 11.30 h after plating onwards. Scale bars measure 10 µM.

To determine whether an $eGFP^+CD14^-$ intermediate stage gave rise to the $eGFP^-CD14^+$ macrophage cells, sorted day 11 $eGFP^-CD34^+CD43^+CD45^+CD14^-$ committed HPC were cultured under myeloid conditions and analyzed using live confocal microscopy to track cells becoming $CD14^+$. We did not observe eGFP expression in cells acquiring CD14 expression (Figure 8 B and Supplemental Movie S2). These data suggest that the generation of $CD14^+$ macrophage cells from hESC is MYB-independent.

Discussion

We here show evidence that multipotent HPCs expressing high levels of MYB are not generated in human EB cultures initiated with hESC. We therefore conclude that the hematopoietic cells generated *in vitro* from hESC using current *in vitro* protocols, emerge through an endothelial intermediate, and are precursors with limited stem cell activity that resemble yolk sac hematopoietic progenitors. In addition we show that the first progenitors derived from endothelial cells are $eGFP^{-}CD34^{+}CD43^{+}$ cells, which then develop into $eGFP^{+}CD34^{+}CD43^{+}$ granulocyte committed progenitors.

Multipotency and self-renewal are two hallmark characteristics of HSC. With regard to multipotency: almost all hematopoietic cell types can be generated from hESC-derived hematopoietic precursors, including erythrocytes(13), megakaryocytes(14), granulocytes, monocytes(15), NK cells(16) and T cells(17, 18). Although, to our knowledge, it has not been demonstrated that a single hESC-derived hematopoietic precursor cell is able to form all of the aforementioned cell types, the absence of these reports may be due to technical issues associated with precursor cells of limited proliferative capacity. The fact that T cells, can be generated from fetal HPC does not in itself prove the presence of multipotent progenitors or HSC, since it has been shown that T cells can be generated from YS precursors that arise before HSC are generated(19, 20). Self-renewal of HSC is evaluated by their ability to reconstitute immune deficient mice. Previous studies have reported repopulation by *in vitro* hESC-derived HPC(21, 22). Wang et al. reported multilineage hematopoietic repopulation upon intrafemoral injection of HPC(22). However, hESC-derived HPCs had limited proliferative and migratory capacity compared with somatic HSCs. In another study by Ledran et al., whole hESC differentiation co-cultures with stromal cells derived from murine fetal tissues were injected intrafemorally in mice. These cells were reported to repopulate bone marrow of the non-injected femur as well as the spleen. In addition, secondary engraftment was reported. However, the engrafted cells were not fully defined. The phenotype shown is compatible with myeloid cells ($CD33^{+}CD13^{+}$). As it is known that tissue macrophages have a long lifespan, and are found lifelong in the adult, without a need for HSC to replenish them, chimerism may have been caused by non-HSC dependent cells. Evidence for engraftment of $CD34^{+}$ cells in the bone marrow was lacking.

In a recent article by Amabile et al. hESC were injected in mice to form teratoma. The authors show that in these teratoma, also hESC derived $CD34^+$ hematopoietic cells are formed. Upon isolation and transplantation of these $CD34^+$ HPC into immunodeficient mice, engraftment of human cells was found, including $CD34^+$ cells in the bone marrow. This *in vivo* differentiation model therefore suggests that HSC can be generated from human pluripotent stem cells (23).

In conclusion, based on these data it remains questionable whether HSC can be formed from ESC using currently available *in vitro* differentiation protocols, although it is clear that multipotent progenitors are formed. Changes to the culture conditions, such as other cytokine mixtures or the use of more appropriate feeder lines may result in HSC generation. The MYB-eGFP cell line that we generated will be very helpful in screening for such conditions. Which factor(s) are missing to generate HSC is unknown to date. However, recent publications using reprogramming of specified cells are starting to shed light on this issue (24-26). Recently it was reported that overexpression of 5 factors in hESC-derived HPC confers short-term engraftment potential to these hESC-derived cells(24). One of the factors which was required for *in vivo* engraftment was characterized as MYB. A similar strategy was used for reprogramming murine hematopoietic cells toward transplantable HSC (26). Although the reprogramming factors needed for conferring HSC properties have not yet reached consensus, this approach may prove to be an alternative method for generating HSC from pluripotent stem cells.

An alternative hypothesis could be that HSC are continuously generated *in vitro*, but lack necessary signals that are present *in vivo*, and thus quickly degenerate to restricted precursor cells, losing their self-renewal capacity. Fetal, newborn and adult HSC are known to lose the capacity to self-renew within days of *in vitro* culture(27-29).

To study the emerging HPC *in vitro*, we isolated $CD34^+$ $CD43^-$ endothelial cells and studied the characteristics of hematopoietic cells generated from them through time-lapse microscopy. We did not observe generation of MYB-eGFP expressing cells, that subsequently lose MYB expression *in vitro*. This is in line with the reported generation of hematopoietic cells directly from hemogenic endothelium from $Myb^{-/-}$ murine ESC, showing MYB to be dispensable for endothelial to hematopoietic transition(30). Instead, we observed either cells that remained MYB negative, or cells,

which up regulated MYB over the course of several days and were further characterized as granulocyte lineage cells. In the murine system, strong evidence was provided that MYB is essential for HSC function. Transplantable HSC were shown to express high levels of Myb (6). This is strengthened by the fact that $Myb^{-/-}$ mice die around fetal day 15 due to lack of transplantable HSC (4), moreover, conditional deletion of Myb leads to exhaustion of the stem cell pool and failure to engraft upon transplantation of LSK cells (6).We can therefore conclude that it is unlikely that HSC are generated in these spin EB cultures.

Conditional MYB knockout models show a marked decrease in granulocytic development, suggesting an important role for MYB in granulocytic development(7). In addition, hematopoietic differentiation cultures set up with $Myb^{-/-}$ murine ESC also showed defective granulocyte lineage differentiation (30, 31). Clarke et al. reported a similar potential to generate CFU-E and CFU-M between wildtype and $Myb^{-/-}$ mESC in short term cultures, however, later on, the numbers of CFU-E and CFU-GM were decreased. Sakamoto et al. compared wild type with $Myb^{-/-}$ mES cells and knock out mES cells that expressed Myb under control of a tetracyclin-inducible promotor. They reported similar numbers of hematopoietic cells generated by wild type and knock out cells, whereas induction of Myb during hematopoietic differentiation resulted in vastly higher cell numbers. Similar to Clarke et al., they report a reduced differentiation towards erythroid and granulocytic lineages, whereas the monocytic differentiation is unaffected. The relative absence of these lineages in mESC cultures reflects the MYB dependency of a lineage restricted erythroid-megakaryocytic and erythro-myeloid precursor rather than the generation of MYB-dependent HSC. These data thus point towards a differential requirement of MYB during macrophage/granulocyte commitment, as is also apparent in our experiments.

Recent studies suggest that tissue macrophages belong to a separate lineage derived from MYB- independent precursors. These are derived from YS derived EMP and persist by local proliferation of terminally differentiated cells or precursors into adult life independent from the bone marrow .The MYB-eGFP- macrophage cells generated in our cultures, co-express CD14 and CD16, a phenotype previously described for tissue macrophages(32, 33).

In conclusion, we have shown that hematopoietic precursor cells that arise from endothelial cells in EB cultures are MYB negative. MYB-positive precursors arise

later in the cultures and are granulocyte lineage restricted. These data therefore provide evidence that bona fide HSC are neither generated nor maintained in these cultures. Rather, yolk sac-like hematopoietic precursors are formed. We here describe that granulocytic lineage committed progenitor diverge thereof, and that this process is accompanied by the upregulation of MYB. In this light reinvestigation of Myb signal in YS precursors might be advised. In addition, we have described a Myb reporter cell line which may be helpful to screen for conditions which can generate HSC from hemogenic endothelium *in vitro*.

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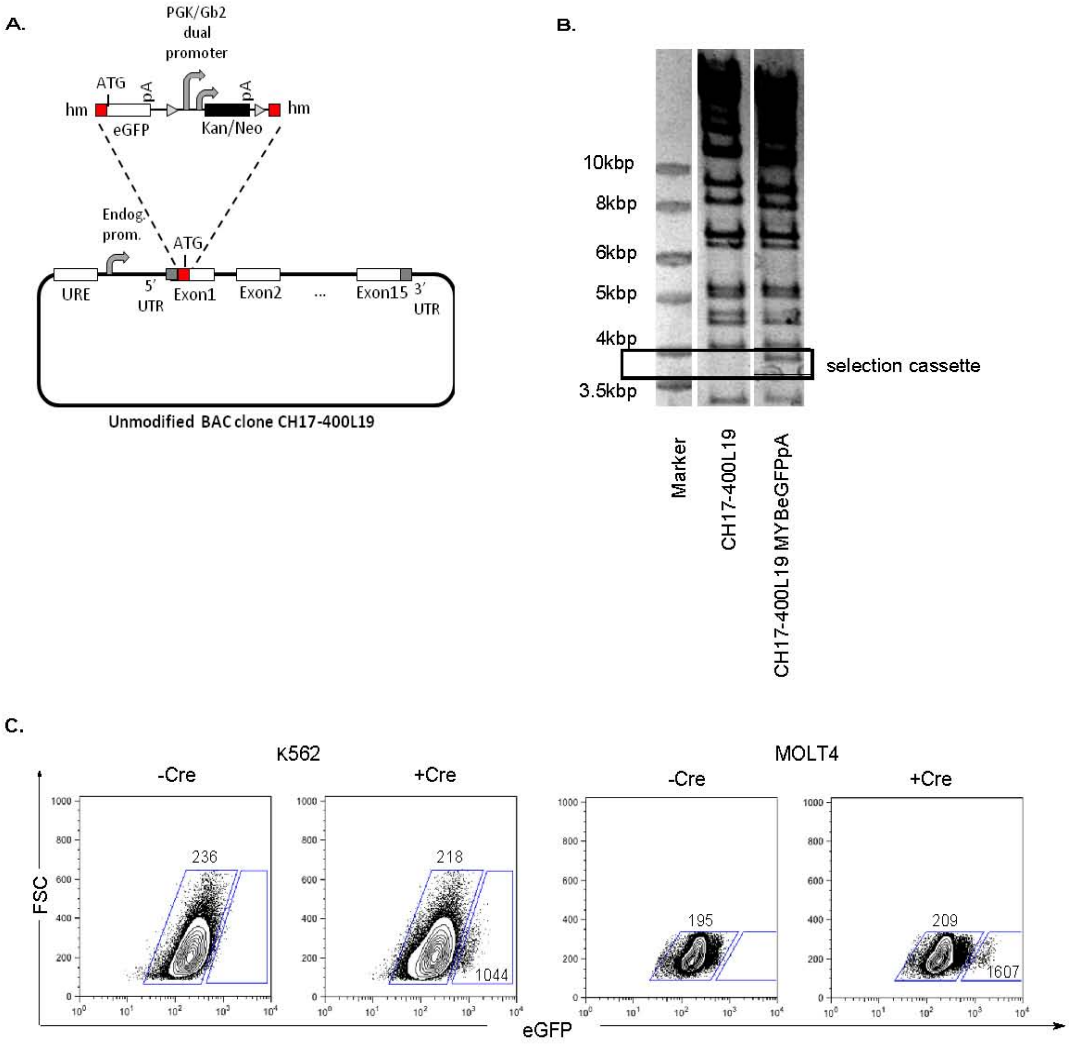
Supplemental data

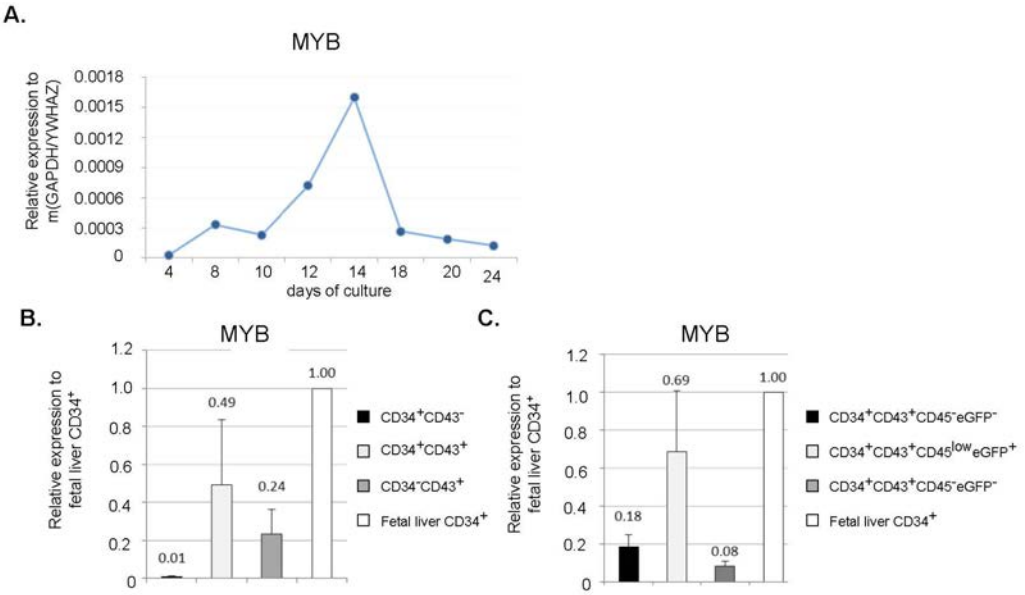
Supplemental table I: primer sequences used in this study

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
MPL	GAGATGCAGTGGCACTTGGA	CCAGATGGGGTACCTGTCTCTG
MYB	GTCTCCAGTCATGTTCCATACC	TGAATGGCTGCGGCAGCT
GATA1	CAAGAAGCGCCTGATTGTCAG	AGTGTCGTGGTGGTCGTCTG
LTF	TGCTGGAGACGTTGCATTTG	CTCZGTCACAGGCTTCCGTT
MBP	TCGGCTCACAAGGGATTCAA	AAGCTGAGGACAGGATTCCG
MPO	TGCATCATCGGTACCCAGTTC	AGATGTTGTTCTTAGACACGGTGG
PU1	GGATCTATACCAACGCCAAACG	GGGTGGAAGTCCCAGTAATGG



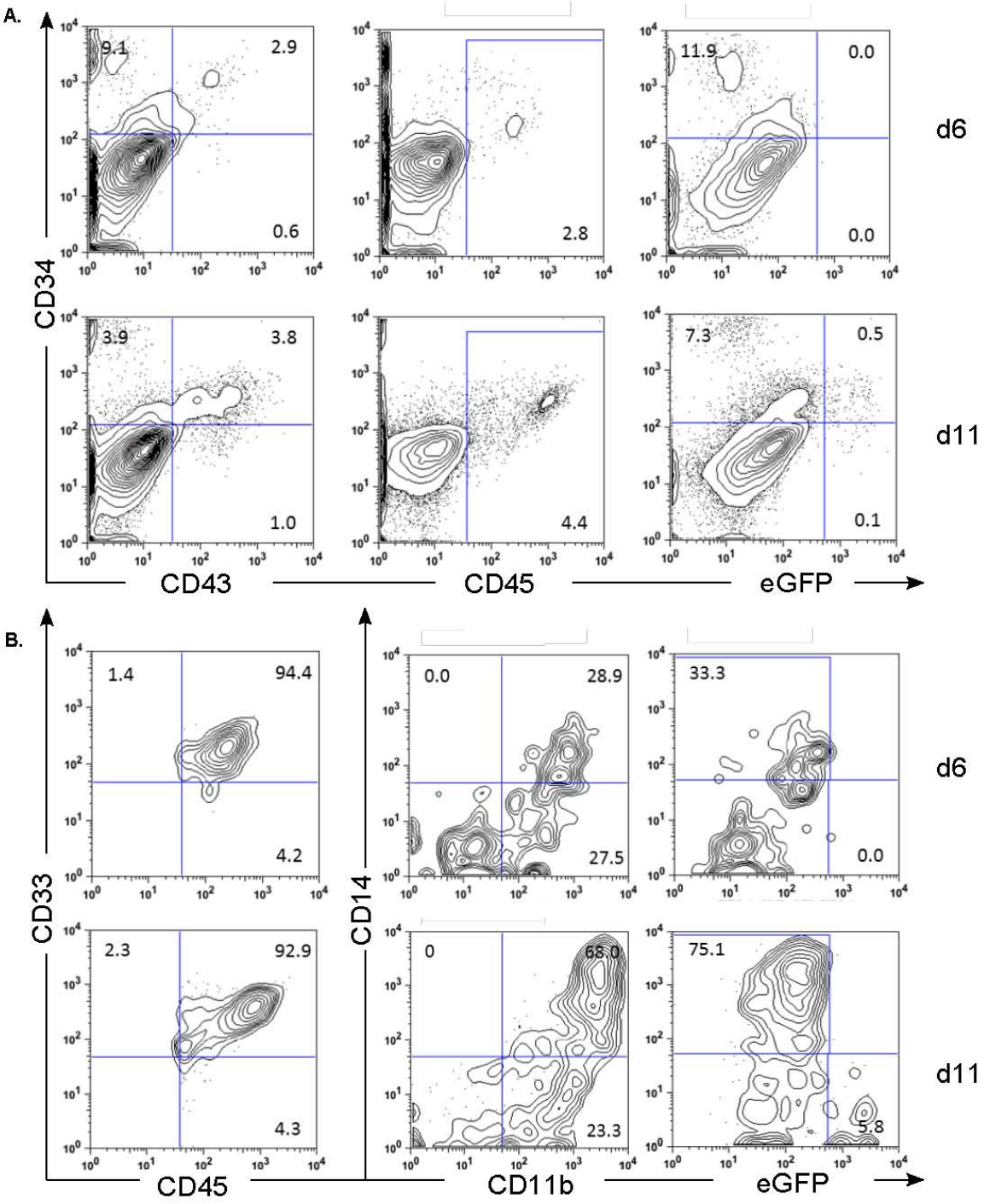
FigureS1: Generation of MYB-eGFP reporter hESC.

A) Schematic overview of BAC recombineering. Red squares indicate homology arms, grey triangles indicate LoxP sequences; B) Restriction digest of the targeted BAC vector shows successful integration of the selection cassette in between BamHI restriction sites; C) K562 and MOLT4 stably transfected with MYB-eGFP BAC plasmid were analyzed for eGFP expression, before and after transient Cre recombinase transfection. Mean Fluorescence Intensities (MFI) for gated populations are shown.



FigureS2: Expression profile of MYB in EB derived hematopoietic progenitors.

A) Kinetics of MYB expression in bulk hESC feeder differentiation culture. Expression is shown relative to the mean of GAPDH and YWHAZ expression; B) MYB expression was analyzed in indicated populations from d14 EB. dCt was calculated relative to the mean of GAPDH and YWHAZ expression. Expression is indicated relative to expression in fetal liver $CD34^+$. Error bars indicate standard deviation of the mean (n=3); C) MYB expression was analyzed in indicated populations within the $CD34^+CD43^+$ population. dCt was calculated relative to the mean of GAPDH and YWHAZ expression. Expression is indicated relative to expression in fetal liver $CD34^+$. Error bars indicate standard deviation of the mean (n=3).



FigureS3: CD34^{+}CD43^{-}eGFP^{-} endothelial cells give rise to eGFP^{-} hematopoietic precursors, before emergence of eGFP^{+}CD34^{+}CD43^{+} cells.

A) Analysis of progeny of CD34^{+}CD43^{-} hemogenic endothelial cells derived from d11 EB. Cells are analyzed after 6 and 11 days of culture respectively; B) Phenotypic analysis of CD45^{+} cells from panel A at indicated time points.

MovieS1: Time-lapse analysis of HPC generation from endothelial cells.

Time-lapse confocal analysis of MYBeGFP hESC d11 EB differentiation sorted $CD34^+$ cells cultured on OP9 with hematopoietic cytokines. Cells were live stained with CD34-APC and CD43-PE. Images were acquired every 30minutes, scale bare measures 100µM, movie is played at a frame rate of 4fps (or 2h per second). Arrow indicates a single $CD34^+$ sorted endothelial cell transforming into a hematopoietic progenitor cells.

MovieS2: Time-lapse analysis of macrophage differentiation.

Time-lapse confocal analysis of MYB-eGFP hESC d11 EB differentiation of sorted $CD34^+CD43^+CD45^+eGFP^-CD14^-$ cells cultured on OP9 with hematopoietic cytokines. Cells were live stained with CD14-PE. Images were acquired every 15 minutes, scale bare measures 100µM, movie is played at a frame rate of 4fps (or 1h per second). Arrow indicates a single progenitor developing into a $CD14^+$ macrophage.

6.2 Pluripotent stem cell based gene therapy for hematological diseases.

Stijn Vanhee and Bart Vandekerckhove

Review article (submitted)

Pluripotent stem cell based gene therapy for hematological diseases.

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Abstract

Gene therapy for inherited hematopoietic disease is currently applied using viral overexpression systems in hematopoietic stem cells. Using retro- or lentiviral vectors, a copy of the functional gene is introduced in hematopoietic stem cells and constitutive expression of the corrected gene is obtained. These transduced hematopoietic stem cells are then transfused back into the patient. While successfully used in the clinic, these methods hold some major limitations both at the safety and efficacy level. Recently, methods to generate patient specific induced pluripotent stem cells (iPSC) from somatic cells were devised. In combination with recent advances in nuclease mediated genetic correction, iPSC technology seems mature for rapid clinical application, especially as gene therapy for hematologic diseases. Combining these technologies, the affected gene could be corrected by replacing the defective gene by a wild type copy in the patient-derived iPSC. These cells can be screened for correct integration and subsequently differentiated towards hematopoietic stem cells or different types of hematopoietic cells. During recent years, the efficiency of genetic modification and *in vitro* hematopoietic differentiation of human PSC has greatly increased. However, protocols for generating *bona fide* hematopoietic stem cells are still lacking. For this reason, we believe that iPSC based gene therapy can be used in conditions where long lived hematopoietic cells mediate the therapeutic effect such as T cells in IL2RG deficiency and tissue macrophages in mucopolysaccharidoses. In this review we give an overview of current protocols and advances in *in vitro* hematopoiesis starting from pluripotent stem cells and evaluate these in the light of gene therapy for hematologic diseases.

Keywords:

Gene Therapy; Hematopoiesis; IPSC; hESC; Pluripotent Stem Cells, Tailored Nucleases, Hematological Diseases; Stem Cells.

1. Genetic correction of hematopoietic stem cells

In 2000, the group of Fischer described the successful genetic correction of the most common form of severe combined immunodeficiency (SCID-X1) by using retroviral overexpression vectors [1, 2]. These patients have an inherited defect in the interleukin 2 common gamma chain (IL2RG gene), leading to a non-functional receptor for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. SCID-X1 patients have low to absent numbers of T cells and NK cells, in the case of an IL-7 or an IL-15 defect respectively. Also, T dependent B cell responses are defective. These defects lead to severe immunodeficiency with recurrent and life threatening infections. Occurrence of such infections starts a few months after birth and does not resolve despite antibiotic therapy. SCID-X1 patients are treated with allogeneic stem cell transplantation, if a HLA identical sibling is available. However, patients without such a donor may be treated with genetically modified autologous hematopoietic stem cells (HSC).

For treatment with genetically modified autologous HSC, $CD34^+$ cells are isolated from bone marrow of the patient. These $CD34^+$ cells are then transduced with a retroviral vector expressing the IL2RG encoding cDNA and reinfused (Figure 1A). This therapy was successful in all patients treated and had similar results to hematopoietic stem cell transplantation from matched siblings. It has to be noted however, that 5 out of 20 patients developed T cell acute lymphoblastic leukemia (T-ALL). This oncogenic transformation was caused by transactivation of *LMO2* or *CCND2* proto-oncogenes. Transactivation was induced by viral integration in close proximity of these latter genes [3-5]. In fact, most of the oncogenic events upon retroviral transduction can be traced back to insertion of the retroviral vector near proto-oncogenes. This poses a particular problem for patients who are especially susceptible to the development of cancer. Increased susceptibility for oncogenic transformation could be detected in aforementioned SCID patients or Wiskott-Aldrich syndrome patients [6].

To alleviate problems related to transactivation of neighboring genes, self-inactivating (SIN) retroviral vectors were used for the treatment of SCID-X1. These SIN vectors lack viral promoter and enhancer activity in their 3′ long terminal repeat (LTR) and cannot activate neighboring genes. In this study, patient derived $CD34^+$ cells were transduced with a SIN vector expressing the IL2RG encoding cDNA. In these

patients successful engraftment of HSC was detected and functional gene corrected peripheral-blood T cells were found in the blood. This thus shows that use of SIN retroviral vectors is an adequate alternative to the use of conventional viral vectors. The use of SIN retroviral vectors may reduce the risk of oncogenic transformation compared to the use of regular retroviral vectors.

Alternatively, SIN lentiviral vectors can be used. Such vectors have the advantage that they readily integrate into the genome of non-dividing cells, in contrast to retroviral vectors. In patients with the Wiskott-Aldrich syndrome such vectors were used for the correction of mutations in the WASP gene [7]. This method has also been used to treat patients with adrenoleukodystrophy (ALD) and β-thalassemia without reports of oncogenic transformation to date [8, 9].

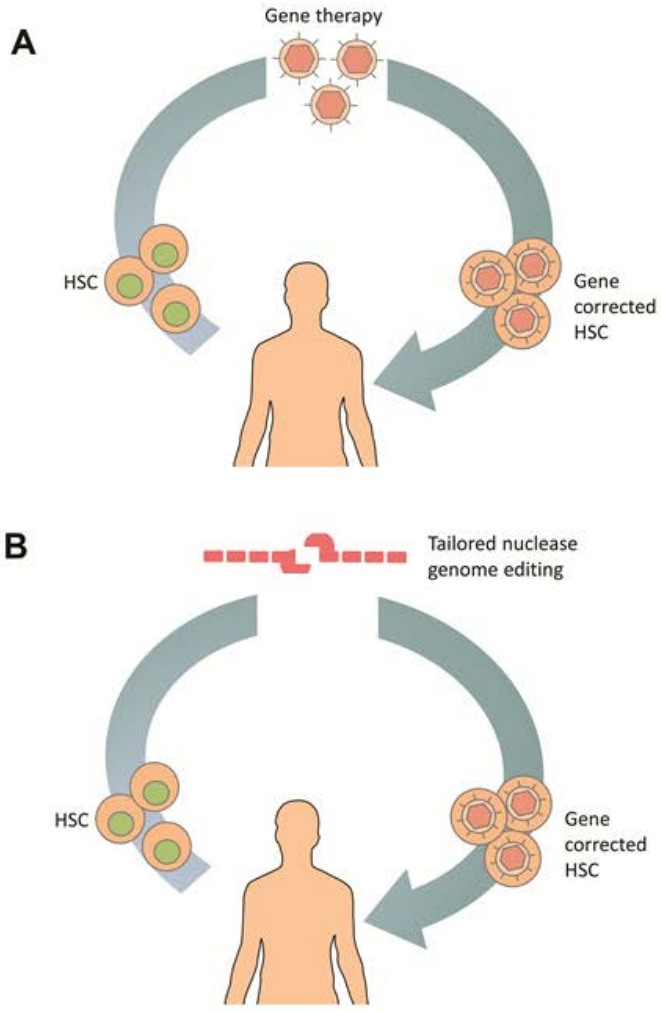

Figure 1: Hematopoietic stem cell based gene therapy for hematological diseases

a) Standard gene therapy using retroviral transduction of hematopoietic stem cells (HSC). Patient derived HSC are transduced with a retroviral expression vector encoding the corrected gene. Upon successful transduction, gene corrected HSC are generated; b) Gene therapy using tailored nucleases to obtain gene corrected HSC.

In conclusion, gene therapy using SIN LTR vectors seems much less likely to induce oncogenic transformation. However, patient follow up is still short. While the LTR used are self-inactivating, integration of the viral vector is still at random. If the viral vector is integrated in regulatory sequences or coding sequences of proto-oncogenes,

insertional mutagenesis is still likely to occur. In combination with clonal selection that occurs during development of T cells, malignant transformation remains a constant threat. Thus, while SIN vectors have been successfully applied in the clinic [1, 7-12], possible adverse effects related to random integration remain a major concern. In addition, expression levels of the corrected gene are not dynamically regulated during differentiation of gene corrected HSC. Constitutive gene expression may in itself enhance malignant transformation, especially in cases where the overexpressed corrected gene is a growth receptor such as the IL2RG.

2. Use of tailored nucleases for genetic correction of HSCs.

During recent years, different genetic engineering methods have made their way into standard laboratory protocols. These strategies all rely on a similar basic strategy, where sequence specific DNA nucleases are targeted to the genomic DNA sequence to be modified. The nuclease associated DNA binding proteins are either Zinc Finger motifs (ZFN)[13], transcription activation like effectors (TALEN)[14] or CRISPR associated proteins (Cas) [15] (for review see *Gaj T, Trends in Biotech, 2013*[16]). Upon successful binding to the target genomic DNA, the associated nuclease will induce a double stranded DNA break (DSB). This DSB is then repaired by the cell in one of two ways: by ligating both ends together by a process called non homologous end joining (NHEJ), alternatively the other DNA strand is used as a template to correct the induced mutation, in a process called homologous recombination (HR). This latter process of HR can be hijacked to introduce the corrected genomic sequence into the chromosome. This hijacking is induced by providing an excess of a “donor plasmid” carrying the corrected DNA sequence to be introduced.

Proof of concept that this method can be applied in the clinic was recently provided by Genovese *et al.[17].* They showed successful gene correction of the IL2RG locus in hematopoietic stem and progenitor cells (HSPC) from SCID patients through HR (Figure 1B). In this study $CD34^+$ HSPC were isolated from patient bone marrow. To induce genetic correction, these HSPC were electroporated with mRNA encoding ZFN targeting the IL2RG genomic DNA after lentiviral transduction to introduce the donor construct. This integrase-deficient lentiviral vector encoded the IL2RG gene

and served as a homologous recombination donor cassette. The authors show that about 3-11% of the HSC successfully integrated the corrected IL2RG gene, as assessed through expression of the associated fluorescent marker that was present in the targeting vector. Off note, less differentiated $CD34^+CD133^+CD90^+$ HSC were found to be targeted with lower efficiency compared to more committed hematopoietic stem cells, suggesting that relatively few long term repopulating stem cells are corrected. In addition, the corrected stem cells are only a minority of the HSC and should therefore be able to successfully compete with non-corrected cells for gene therapy to be successful.

The use of tailored nucleases for clinical applications is a relatively new technology and thus warrants thorough safety analysis. To this end, both efficiency and specificity of the tailored nucleases are of utmost importance. The use of the ZFN method in *ex vivo* HSC was found to be highly efficient in this study [17]. Upon targeting with ZFN, insertion or deletion of bases (indel) in the IL2RG locus were found in around 50% of the cells. This high rate of indels shows that the IL2RG specific ZFN nuclease targets the IL2RG locus with high efficiency. However, homologous recombination is the limiting factor for efficient integration of the corrected gene since only 3-11% of the cells were corrected by homologous recombination. To evaluate ZFN specificity, targeting of potential off-target sites of these ZFN was addressed. Upon sequencing of these presumptive off-target sites the rate of mutation was found to be about 0.7-1.7%. This proves the high specificity of the ZFN used, with potential off-target sites being targeted with only low efficiencies. Although successful gene correction efficiencies are low, in the case of IL2RG correction in the HSPC, T and NK cells derived from corrected HSPC are expected to have a selective advantage over their non-corrected counterparts [17].

Although this study is promising, genetic modification of human stem cells still remains far from being routine practice. Successful genetic modification using tailored nucleases depends on a number of limiting prerequisites: 1) efficiency of transfection and adequate survival of the cells during the transfection process, 2) successful homologous recombination and 3) the availability of an in vivo or in vitro method for selection of the corrected cells.

The first two require active cycling of the targeted cells, which poses a major problem, as HSC are quiescent and do not readily divide. Induction of HSC cycling requires

addition of *ex vivo* cytokines. This causes HSC to commit to hematopoietic lineages and differentiate, reducing their use for transplantation.

With newer technologies, efficiencies of nuclease genome editing are readily increasing. The main problem for gene correction thus remains the low efficiency of homologous recombination in non-cycling human cells. In the case of X-SCID the gene-corrected cells are supposed to have a selective advantage *in vivo*, however this is not the case for other diseases such as WAS or β-thalasemia. In those cases where the corrected gene does not impose a selective advantage, corrected HSC would need to be selected *in vitro*. Such methods generally rely on selection using an antibiotic for several days, or cell selection is based on a co-expressed membrane marker. *Ex vivo* selection inevitably requires prolonged culture, which would further cause differentiation of the corrected HSC.

Another major drawback is the impossibility to screen for off-target mutations or integrations. As shown, such off-target effects do not occur with high efficiency, but hold oncogenic potential nonetheless [17].

3. Advances in generation and genetic modification of pluripotent stem cells for clinical applications.

The group of James Thomson first derived human embryonic stem cells (hESC) in 1998 [18]. These hESC are derived by culture of human pre-implantation embryos [19]. hESC possess the ability to generate virtually every cell type of the human body, a characteristic termed “pluripotency”. Pluripotency is evidenced by the generation of teratomas, which are generated upon injection of hESC in immune deficient mice. These teratomas contain cells derived from all three germ layers, and thus prove the pluripotent properties of hESC [18]. Another hallmark characteristic of these cells is their ability to be propagated indefinitely *in vitro*. This latter characteristic thus allows unlimited expansion of hESC without differentiation.

hESC, however, remain topic of ethical debate and are of low applicability in the clinic as these are allogeneic to the patient. With the description of human induced

pluripotent stem cells (hiPSC) generation, the group of Shinya Yamanaka revolutionized the field by showing that fully differentiated adult cells can be "reprogrammed" towards fully pluripotent cells [20]. iPSC cells show close similarity to hESC on transcript level, on the morphological level and have similar properties as do hESC. Similar to hESC, hiPSC are pluripotent and can be expanded indefinitely during *in vitro* culture [20]. hIPSC were initially generated by reprogramming of fibroblasts obtained from skin biopsy. In these reports, reprograming was obtained by retroviral transduction of the fibroblast cells with only four factors: Oct3/4, Sox2, Klf4 and c-Myc. Recently, less invasive sources than fibroblasts obtained through skin biopsy have been used for somatic cell reprogramming. Among these cell types are circulating white blood cells and hematopoietic progenitor cells (HPC) derived from cord blood and adult peripheral blood [21-26].

In the Yamanaka paper retroviral vectors were used for somatic cell reprogramming. These integrating retroviral vectors contain known oncogenes such as *cMyc*. This may render reprogrammed cells prone to oncogenic transformation. To alleviate these safety issues, alternative reprogramming strategies were developed. Instead of using integrating viral vectors, successful reprograming was obtained using non-integrating techniques. Among these techniques are transfection of mRNA [27], transfection of protein [28] or transduction with non-integrating Sendai viral particles [29]. All these methods circumvent the use of potentially oncogenic viral integration techniques.

Patient derived iPSC can be corrected using the aforementioned methods. As iPSC can be cultured indefinitely, the potential gene corrected iPSC can be easily screened and clonally expanded. This allows for exclusion of cells where off-target effects occurred, and would even allow for functional assessment of the gene correction in iPSC-derived cells upon differentiation.

As iPSC can be derived without the need for donor human embryos, ethical issues have become less of a problem. iPSC technology enables us to generate patient derived autologous pluripotent cells, which can be differentiated to every cell type of the human body. The use of autologous cells could alleviate immunologic issues upon transplantation, at least for acute rejections [30]. For clinical purposes, hiPSC can be derived from the patient itself, or, alternatively allogeneic histocompatible iPSC could be obtained from a public iPSC bank [31]. Such a public iPSC bank may

circumvent the need for reprograming, correction and screening of patient derived cells, although in this case it is likely that lifelong immunosuppressive therapy should be prescribed.

In conclusion, it is currently possible to generate patient specific pluripotent stem cells (PSC). These can be derived from starting material obtained by minimally invasive methods, such as peripheral blood, cord blood or skin biopsies. iPSC could then be subjected to genetic correction and differentiated towards HSC or specific types of blood cells suited for transplantation (Figure 2).

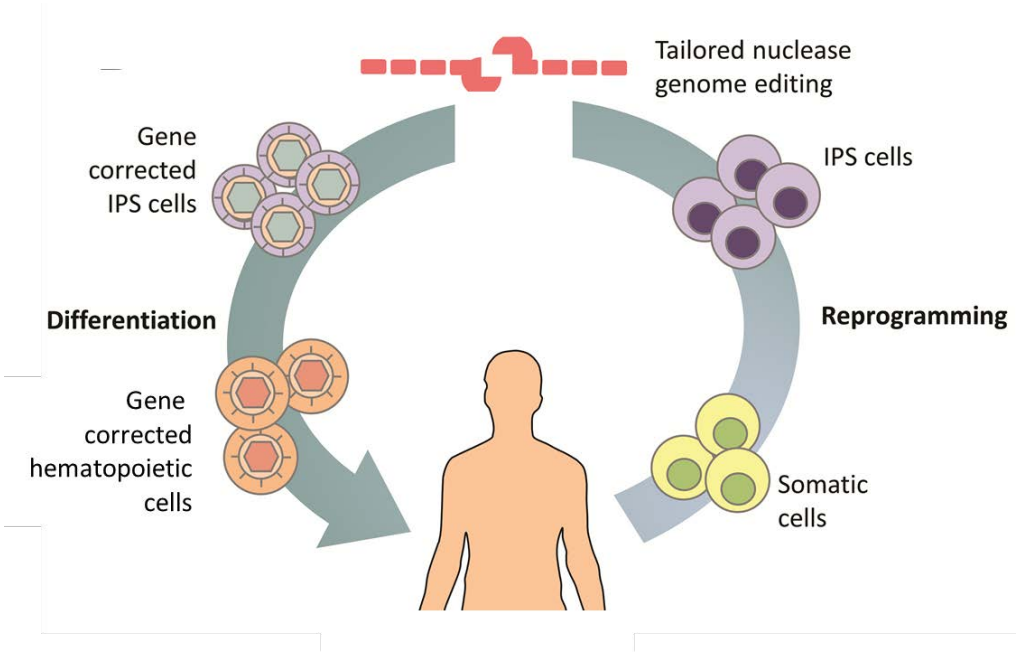

Figure 2: Pluripotent stem cell based gene therapy for hematological diseases

Gene therapy using tailored nucleases to obtain gene corrected hematopoietic cells derived from patient specific pluripotent stem cells. Somatic cells are reprogramed towards induced pluripotent stem cells (iPSC), in which the affected gene is then corrected. After in vitro hematopoietic differentiation of iPSC, the obtained cells can be transfused back to the patient.

4. Pluripotent stem cell derived blood cells

During the last decade, different protocols to generate blood cells from PSC *in vitro* have been described [32-38]. In general, two types of strategies are used to initiate hematopoiesis from pluripotent stem cells: embryoid body (EB) based and stromal cell line (feeder) based differentiation methods. The former method relies on the aggregation of small fragments of PSC or a single cell suspension of PSC into a small clump. This re-aggregated clump is differentiates into an “embryoid body” and contains PSC differentiated to different cell types. The latter method relies on transfer of fragments of pluripotent stem cells onto a feeder layer. Generally feeder cells of animal origin are used in combination with media containing components of animal origin. Use of xenogeneic feeder cells renders the PSC-derived cells inadequate for use in patients. In addition, the efficiency of blood cell generation remains low in most of these protocols, with only a few percent of input cells leading to hematopoietic cells. Of these differentiated cells, cells expressing $CD34^+$ or $CD45^+$ are generally found with frequencies between 1 -50%.

For patient use, differentiation protocols are needed which are devoid of any component of animal origin. This can be obtained by omitting xenogeneic feeder cells, using serum-free medium and using recombinant growth factors. To date, few of these so called “xeno-free” methods have been described [35, 37, 38]. Of these protocols, the ones described by the group of Andrew Elefanty appear promising. With this method about 1:500 of the input pluripotent cells generates cells with hematopoietic potential. These cultures give rise to over 20% $CD34^+$ cells. However, it should be noted that this population is not a pure hematopoietic progenitor cell population. By the end of culture, around 30% of the cells express CD45. In this protocol, a fixed number of single-cell adapted hESC are differentiated towards hematopoietic cells using the EB methodology. These re-aggregated hESC clusters, are then grown for up to 12 days in xeno-free APEL-medium supplemented with SCF, BMP4 and VEGF. Addition of extra factors such as APELIN, further increases the hematopoietic differentiation efficiency, but also induces rapid differentiation of hematopoietic progenitors [38]. The upside of this method is that it is highly scalable and differentiation is consistantly induced with limited variability.

In mice, successful *in vivo* reconstitution of irradiated mice with murine PSC-derived hematopoietic progenitors was shown after transduction of these hematopoietic precursor cells with HOXB4 [39]. This led to the belief that HSC generation from PSC for clinical purpose should be possible.

As addressed earlier, PSC have the inherent ability to form any cell type of the body *in vivo*. Also *in vitro*, many hematopoietic cell types have been generated from hESC and hIPSC. NK cells [40], T cells [41, 42], megakaryocytes [43], erythrocytes [44], monocytes and granulocytes [45] have all been generated from PSC *in vitro*. However to date the generation of a *bona fide* HSC from PSC has not been reported unequivocally. The gold standard for the demonstration of human HSC properties such as self-renewal remains the repopulation assay in immune deficient animals. This assay was developed by John Dick to test the hematopoietic properties of postnatal hematopoietic cell populations [46]. For this assay a HSC containing suspension is injected intravenously or intrafemorally in sublethally irradiated recipients. Upon successful engraftment these animals have human $CD45^+$ cells in the blood and bone marrow for up to 12 weeks. Human cells consist of myeloid cells, B cells and NK cells. In addition, human cells repopulate the thymus and generate human T cells. Secondary transfer of bone marrow cells from these repopulated mice into novel recipient mice should again result in repopulation.

However, when testing fetal or ESC-derived populations for HSC activity, care has to be taken that long lived cells other than HSC derived cells do not interfere with the assay. For this reason, we think that this assay should show: 1) chimerism in the blood or spleen of these animals, 2) the human cells consist of at least erythroid, myeloid and lymphoid lineages, and 3) the bone marrow should contain human $CD34^+$ cells which upon purification and secondary transfer give rise to multilineage reconstitution.

The first reports showing generation of HPC from hESC capable of SCID repopulation were published by the groups of Mickie Bhatia [47] and Dan Kaufman [48]. Both these groups have shown successful *in vitro* differentiation of hESC towards hematopoietic cells, and in addition, repopulation *in vivo*. However, both also show clear differences between these hESC-derived multipotent progenitor cells and somatic HSC.

In the publication by the group of Mickie Bhatia [47] the CFU progenitor frequencies were reported to be similar between somatic HSC and hESC-derived (HPC). However, the hESC-derived HPC showed a skewed CFU potential towards granulocytic cells as compared with the potential of somatic HSC. To test HSC properties of the hESC derived hematopoietic progenitors, cells were intravenously injected into immune deficient mice. After injection approximately 60% of mice died due to formation of lung emboli, induced by factors in murine serum. As somatic HSC do not cluster in these same conditions, this is another indication for differences between both types of progenitor cells. Using flow cytometry, the hESC-derived stem cells clearly show higher expression of CD45 and display a higher granularity.

Intrafemoral injection of the hESC-derived HPC cells alleviates the problem of lung emboli formation, and resulted in engraftment of human cells. The authors show the presence of human cells in the bone marrow after injection, although it is not clear how long they remain detectable after injection. These cells show expression of CD33 and CD19, which points towards lineage commitment. However, the CD34 expression profile of the successfully engrafted cells is not shown. It has to be noted that engraftment remains low in bone marrow compared to somatic HSC (10-100 fold lower). Interestingly, when gene expression was analyzed and compared between somatic HSC and hESC-derived HPC, clear differences were shown. Main differences were found in clusters regulating cell replication, transcription, cell-cell contact and migration. Also the pattern of homeobox (HOX) genes was markedly different, further indicating the differences between both types of HPC [47].

The group of Dan Kaufman [48], reported similarly low levels of engraftment with hESC-derived HPC (~0.1-1%). Engraftment was more pronounced when recipient NK cells were largely eliminated by treatment with anti-ASGM1 antiserum. The authors were able to show expression of human CD34 on cells injected into the bone marrow, but no evidence is provided for multilineage repopulation. These publications also fail to show successful secondary repopulation of the hESC derived HPC.

In a publication by the group of Majlinda Lako [33], hESC are differentiated towards hematopoietic cells on different stromal cell lines derived from murine urogenital ridges, aorta-gonado-mesonephros region (AGM) and fetal liver (FL). The AM20.1B4 cell line, derived from the AGM region, was best in supporting the generation of

hematopoietic cells from hESC capable of engraftment. Upon intrafemoral injection of hESC derived progenitors an average engraftment in the contralateral femur was found to be around 2%. In the peripheral blood human cells were 16% of the total of hematopoietic cells. The cells found in the peripheral blood and bone marrow expressed CD33, CD13, CD2 and CD19 and were high in forward scatter, showing the large size of these cells. The authors remark themselves that despite the presence of lymphoid markers (CD2 and CD19) they cannot exclude that these are myeloid committed cells.

Taken together, these reports do not show solid evidence for the presence of HSC in PSC derived cultures. The long term presence of human cells was demonstrated, however no multilineage reconstitution was shown, nor is there solid evidence for engraftment of $CD34^+$ cells in the bone marrow responsible for primary and secondary reconstitution. It is possible that these long-term surviving human cells are contaminating non-hematopoietic cells or HSC independent myeloid cells such as tissue macrophages.

In a recent publication by Amabile et al. undifferentiated hESC were injected in immunodeficient mice. Here these hESC readily form teratomas, containing cell types of all germ layers. The authors show that in these teratomas also hESC derived $CD34^+$ hematopoietic cells are formed. These develop under the influence of factors originating from the injected hESC or from the recipient mouse. Upon isolation and transplantation of these $CD34^+$ cells into immunodeficient mice, engraftment of human hematopoietic cells was found. These cells also hold multilineage reconstitution capacity, but it has to be noted that they show lineage skewing towards the myeloid lineage after transplantation. This is in contrast to CB derived $CD34^+$. While these data support the inherent pluripotent potential of hESC, the factors leading to the generation of HSC remain unexplored and elusive. Further research on these factors will certainly provide more insight in the complex ontogeny of the hematopoietic system.

The properties of HPCs formed in these cultures remain poorly understood. Expansion of the PSC-derived $CD34^+$ population without apparent commitment, would be an excellent way to remediate the low cell numbers generally obtained. However, while able to replicate in a burst like fashion, expansion of these PSC-derived cells is rapidly exhausted. Upon expansion, the HPC rapidly commit to the

erythroid or myeloid lineage and give rise to terminally differentiated cells. In addition, the lack of successful engraftment and multilineage reconstitution capacity of pluripotent stem cell derived HPC thus hampers the use of these generated $CD34^+$ cells for direct transplantation in a clinical setting. To understand the generation and properties of progenitor cells in vitro, it is essential to understand the ontogeny of the hematopoietic system in the embryo.

5. *In vivo* murine hematopoiesis

In the embryo, hematopoiesis occurs at different locations, both extra-embryonic and inside the embryo. During decades of research, hematopoiesis has been conceptualized as the formation of the hematopoietic system in largely two distinct waves: a first “primitive” wave and a second “definitive” wave. During the brief wave of primitive hematopoiesis the developing embryo forms blood cells in the yolk sac. All hematopoiesis beyond this wave is termed “definitive” hematopoiesis. During definitive hematopoiesis, blood cells are generated at different sites, both extra-embryonic (in the yolk sac or YS) as intra-embryonic (in the aorta-gonado-mesonephros or AGM region). After these waves hematopoiesis shifts to other, more specialized hematopoietic organs. HSC generated in the AGM, will migrate to the fetal liver, where they will mature and vastly expand. These mature HSC will then populate the bone marrow, where they sustain lifelong hematopoiesis (Figure 3A).

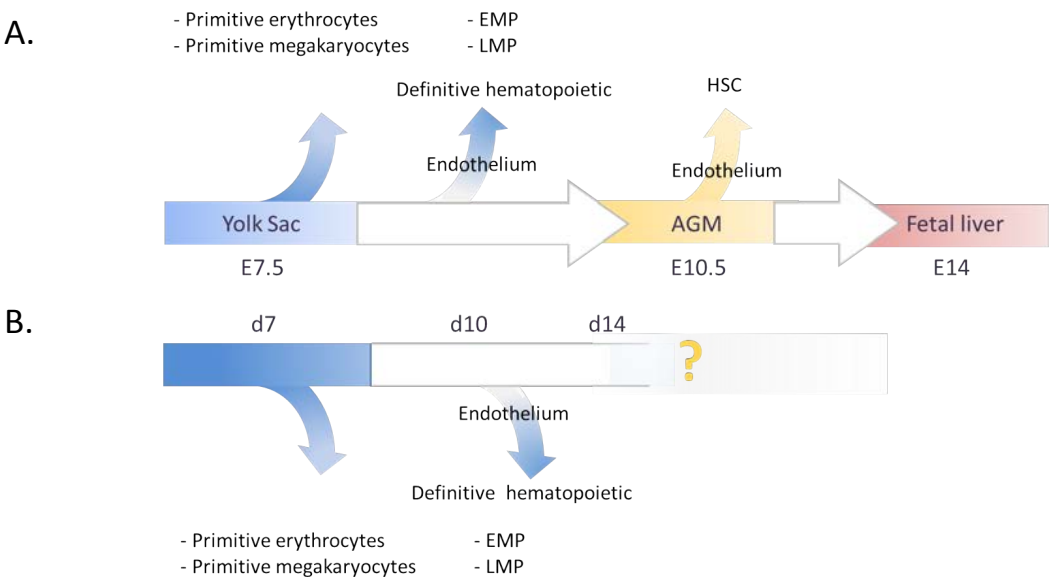

Figure 3: schematic overview of hematopoiesis

a) Ontogeny of the hematopoietic system in the mouse. A first wave of primitive hematopoiesis occurs in the yolk sac at embryonic day 7.5 (E7.5). Here primitive erythrocytes and megakaryocytes are generated. Subsequently a definitive wave of hematopoiesis is initiated between E8.5 and E9.5, during which erythro-myeloid progenitors (EMP) and lympho-myeloid progenitors (LMP) are formed. Hematopoietic stem cells (HSC) are generated in the embryo at E10.5 in the aorta-gonado-mesonephros (AGM) region. These cells will then migrate to the fetal liver, where they further develop and expand; b) Development of hematopoietic cells in vitro from human embryonic stem cells. In analogy with the in vivo situation, during a first primitive wave after 5-7 days of differentiation, primitive erythrocytes and megakaryocytes are generated. Later on in culture, between day 10 and 14, erythro-myeloid progenitors (EMP) and lympho-myeloid progenitors (LMP) are generated. Whether these cultures proceed to the generation of an AGM-like hematopoietic process has not been established.

5.1 Different waves of hematopoiesis

5.1.1 Primitive yolk sac based hematopoiesis

During the development of the embryo, oxygenation of tissues by diffusion is unable to cope with the increasing need for oxygen during growth. At this time, the embryo is in urgent need of a functional cardiovascular system carrying oxygen to the developing organs. To this end a primitive hematopoietic system is triggered to form immature cells, which are able to sustain oxygenation of the tissues.

The first hematopoietic cells that are formed in the extra-embryonic tissues around E7.5 are in close proximity of the endothelial cells lining the “blood islands” of the YS. These first, primitive, blood cells generated within the blood islands are marked by their large size and expression of both embryonic and fetal hemoglobin chains [49]. During primitive hematopoiesis no hematopoietic stem cells are formed. This concept was established by determining that at E7.5-E8.25 no transplantable HSC can be derived from the YS, nor are multipotent progenitors generated at this timepoint [50] (Figure 3A).

5.1.2 Definitive yolk sac based hematopoiesis

After the primitive wave of hematopoiesis, the definitive wave of hematopoiesis is initiated. This wave occurs in both YS and AGM/para-aorta-splanchno pleura (PAS) regions. During early definitive hematopoiesis, the YS gives rise to multiple types of hematopoietic (progenitor) cells, which do not show HSC properties. In zebrafish and mice, these multipotent cells have been termed erythro-myeloid precursors (EMP) and give rise to erythroid cells and all types of myeloid cells [51] and reviewed in [52] (Figure 3A).

E8.5 YS-derived progenitors give rise to cells of the mononuclear phagocyte system (MPS). The monocytes generated here sustain lifelong and are independent of a bone marrow HSC. Recently Schulz and colleagues have shown that two parallel pathways of macrophage differentiation can be detected. These parallel pathways

can be distinguished based on their differential dependence on MYB [53]. The cells of the MPS system originate in the YS from MYB independent precursors. These precursors give rise to tissue macrophages such as liver Kupffer cells and skin Langerhans cells. This is in contrast with HSC based hematopoiesis, which is strictly MYB dependent. Ginhoux *et al.* have shown in a similar fashion that microglia have an origin in E7.5 YS hematopoiesis which is HSC independent [54].

Recent reports also show that E9.5 YS definitive hematopoiesis contributes to lymphopoiesis (Figure 3A). This suggests the existence of a possible lympho-myeloid restricted precursor (LMP) already before the initiation of AGM HSC based hematopoiesis. In the publication by Yoshimoto et al. [55] evidence is presented for lymphoid potential in both YS and para-aortic-splanchno pleura (PAS) at E9.5, before emergence of HSC at E10.5. To further strengthen the fact that lymphoid cells are autonomously generated in the YS, experiments were performed using $NCX1^{-/-}$ mice, which lack active circulation. This excludes passive migration of AGM-derived HSC to the yolk sac. It was shown that cells expressing VE-Cadherin (marking endothelium) and lacking CD41 (marking the first hematopoietic cells) were the precursors of these YS-derived T cells. T precursor cells derived from this YS endothelium were able to reconstitute fetal thymus and were found to be functional. Moreover, these cells gave rise to both TCRγδ and TCRαβ T cells, which were of polyclonal nature. It has to be noted that in this report lymphoid progenitors cells were generated *in vitro*. The observation that lymphoid progenitors could be found prior to the emergence of HSC *in vivo* was studied in more detail by Böiers *et al* [56]. The authors use a Rag1-GFP reporter mouse to trace the earliest cells having lymphoid potential. These cells were defined as being $Lin^{-}Kit^{+}Flt3^{+}IL7Ra^{+}$, and were found in the YS as early as E9.5. At this timepoint the embryo proper lacks lymphoid potential. These cells were found to be $CD45^{+}$ and in contrast to the report by Yoshimoto *et al.*, these co-expressed CD41. The cells reported by Böiers *et al* do not show MegE potential, but are able to form T, B and NK cells. Also these cells seem to contribute to myeloid lineages. As these cells are found as early as E9.5, this excludes their origin in AGM derived HSC. However, it remains to be defined whether these cells derive from multipotent YS progenitors or whether they originate as lymphoid lineage restricted progenitors directly from hemogenic endothelium in the YS, as suggested by the data of Yoshimoto *et al*. [55]

Together, these recent data thus underscore the previously under-appreciated potential of HSC independent YS based hematopoiesis.

5.1.3 Definitive AGM based hematopoiesis

The hematopoietic stem cell is first detected in the AGM region. The “true” hematopoietic stem cell, capable of reconstitution of an adult mouse, is formed here at a later time point than the onset of definitive hematopoiesis in the YS (Figure 3A). Medvinsky and Dzierzak [50], using explant cultures, elegantly showed that HSC formation is initiated in the E10.5 AGM region. At this time point the YS lacks any transplantable activity. Immature HSC are formed in the ventral aspect of the E10.5 AGM directly from the endothelial lining, a concept also found in other model organisms such as the zebrafish [57, 58]. However, HSC generated in the AGM do not directly acquire their full potential, but first require functional maturation and expansion in the fetal liver [59]. These mature HSC will then seed the spleen and bone marrow to sustain lifelong HSC-dependent hematopoiesis. The embryo becomes dependent on HSC-dependent hematopoiesis around E15. Deficiencies in HSC specific genes such as *Myb* and *Meis1* are lethal around E15 [60, 61]. This is in contrast with hematopoietic specific genes such as *Runx1* and *Lmo2* which are lethal around E9-11, when the embryo becomes dependent on the blood circulation for oxygen transport [62, 63].

Adding to the complexity of HSC biology, fetal-type HSC are markedly different from mature adult HSC. From FL hematopoiesis at ~E14 until 3-4 weeks after birth in the bone marrow, fetal HSC can be isolated which have higher proliferative activity and can give rise to specific types of γδ-T cells and B1 marginal B cells [64, 65]. These cells seem to be regulated by a, to date unraveled, specific genetic program which gradually shifts towards the mature HSC program and tips over between week 3 and week 4 after birth. In this process Lin28b has been shown to be a key regulator of the fetal HSC program [66].

Thus, it is clear that hematopoiesis is a complex and stepwise system, giving rise to multiple progenitor cells with different capacity arising at different time points and locations. The molecular mechanisms underlying these steps still remain to be unraveled. Currently, the only true functional test of the generated cells remains assessment of their functionality, by engraftment or *in vitro* multi-lineage differentiation.

Acknowledging the temporal changes during *in vivo* hematopoiesis, starting with a wave of primitive hematopoiesis in the yolk sac, followed by a wave of definitive hematopoiesis in the yolk sac and finally a wave of HSC formation in the AGM, one might hypothesize that *in vitro* hematopoiesis from hESC may follow a similar path (Figure 3B).

6. Generating pluripotent stem cell derived HSC – where are we now?

Based on the data mentioned above, it is unclear whether HSC are generated in PSC differentiation cultures. Alternatively, it is possible that HSC do arise but rapidly disappear or differentiate due to lack of supportive culture conditions.

To address this question, we generated a MYB reporter hESC line, to mark emergence of MYB dependent HSC in the *in vitro* cultures [67]. The use of a *MYB* reporter line, allows for discrimination of YS MYB-independent hematopoiesis or fetal liver HSC-based MYB dependent hematopoiesis. We have shown, using this MYB-eGFP reporter hESC line that the generation of blood cells from hemogenic endothelium occurs without the progression through a MYB-eGFP positive stage. The first $CD34^{+}CD43^{+}$ MYB-$eGFP^{+}$ cells occur relatively late in these cultures, around day 14. These cells express a $CD34^{+}CD45RA^{-}CD38^{-}CD49f^{+}$ stem cell phenotype, however, the myeloid surface markers CD33 and CD123 are also expressed by these cells. Adding to this, these cells are readily expandable in media containing IL-3, further suggesting myeloid commitment. Using rt-qPCR, we were able to show commitment of the $eGFP^{+}CD34^{+}CD43^{+}CD45^{-/lo}$ populations towards the

granulocytic myeloid lineage, through expression of high levels of *MPO* and *GATA1*. This was reflected in their lineage potential, as these were limited to generate granulocytic committed myeloid cells. The first multipotent hematopoietic cells generated in these cultures therefore do not pass through a *MYB* positive stage. $CD34^+$ cells expressing this marker show commitment towards the granulocytic lineage rather than multipotency. Upon closer investigation of the monocytic cells generated here, we were able to show that these cells are reminiscent of tissue macrophages. *In vivo*, these tissue macrophages derive from MYB independent YS precursors, without dependence of HSC, as discussed earlier. Between day 10 and day 12 of culture, lymphoid precursors that give rise to T and NK cells also arise. This thus suggests that these cells are also formed from a MYB-eGFP yolk sac ELP-like cell.

Based on our experimental results, we hypothesize that *in vitro* hematopoiesis from hESC mimics definitive YS EMP and LMP based hematopoiesis and that *bona fide* HSC are not generated (Figure 3B).

Although reports have published the generation of $CD34^+$ stem cells from hESC capable of engraftment, there is no solid proof for *in vitro* generation of HSC based on functional assays. As evidence is accumulating that HSC are not formed *in vitro* using current differentiation protocols, it is becoming clear that the goal of generating transplantable HSC from pluripotent stem cells for clinical practice is not yet achieved. Using reporter cell lines, such as our MYB-eGFP hESC reporter line, experimental conditions could be identified which do generate MYB^+ progenitor cells with HSC properties during *in vitro* pluripotent stem cell differentiation culture.

7. Towards clinical application of human pluripotent stem cells – the road ahead.

As we have discussed, generation of bona fide HSC from PSC has not been successful to date, however current PSC differentiation methods do allow for generation of patient specific cells that may be suitable for transplantation (figure 4).

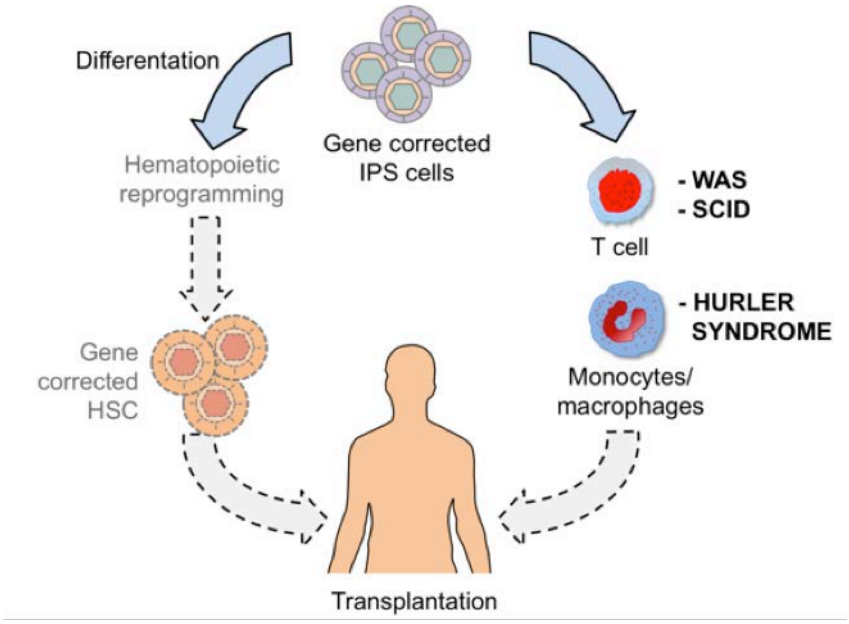

Figure 4: Generation of hematopoietic cells from pluripotent stem cells with clinical applicability.

Gene corrected induced pluripotent stem cells (iPSC) derived blood cells can either be reprogrammed to generate gene corrected induced HSC (iHSC) and transfused into the patient, alternatively, iPSC can be directly differentiated towards end cells for transplantation. Suitable cases would be the transplantation of gene corrected T cells for treatment of Wiskott-Aldrich syndrome (WAS) or severe combined immunodeficiency syndrome (SCID) and transplantation of macrophages for the treatment of Hurler syndrome

We were the first to show that hESC differentiation cultures give rise to mature and functional T cells [41]. *In vitro* differentiation culture of PSC to T cells could be applied for lymphoid cell diseases, such as the aforementioned X-SCID and Wiskott-Aldrich syndrome. T cells are long lived, and vastly expand during development and upon recognition of their ligand. Thus transplantation of gene corrected T cell precursors generated *in vitro* might provide a sufficient basis for reconstitution of T cell immunity.

Another type of long-lived YS-like cells that can be generated from PSC are tissue macrophages, as we have shown [67]. These cells may be ideal for transplantation in

the case of mucopolysaccharidoses (MPS) such as MPS I. MPSI, also known as Hurler syndrome, is a multisystemic disease caused by accumulation of carbohydrate polymers [68]. One of the affected cell types are tissue macrophages, among which are liver Kupffer cells and brain microglia [69]. As these cells are long lived and are easily generated from pluripotent stem cells, transplantation of patient specific gene corrected macrophage precursors could provide a highly efficient and safe alternative therapy to transplantation of hematopoietic stem cells, especially to prevent central nervous system complications.

However, for broad applicability of PSC technology for the treatment of inborn hematologic diseases, generation of functional HSC is required.

Advances towards generation of HSC from PSC will inevitably be made in the coming years based on increased understanding of *in vivo* hematopoietic processes. Knowledge of the factors, which are essential to induce definitive hematopoiesis at the different time points and anatomical locations during development, can be translated to the *in vitro* system. While no formal proof for HSC formation from hESC has been provided, knowledge of *in vivo* hematopoiesis has greatly advanced in vitro hematopoietic differentiation.

One of the first reports regarding hematopoietic induction from hESC, was by Chadwick et al [34]. Here it was shown that upon addition of hematopoietic cytokines, a mix containing SCF, Flt3-L, IL-3, IL-6 and BMP4, generation of hematopoietic (progenitor) cells was greatly increased. The percentage of $CD34^+$ cells was increased by almost twofold by addition of these cytokines, while total cell numbers remained stable. This suggests increased efficiency of hematopoietic differentiation. Also the number of CFU was vastly increased.

Woll *et al.* [70] describe both increased kinetics of differentiation upon addition of Wnt1 containing conditioned medium to these cultures. The percentage of hemato-endothelial cells was also increased. However, the authors used differentiation protocols using stromal cells and undefined serum component, hampering direct conclusions about the effect of Wnt1 signaling itself. In a more recent paper by Gertow *et al*.[71] the effect of Wnt signaling was addressed in defined media conditions. Here the authors conclude that Wnt3a signaling in itself is insufficient for successful induction of mesodermal differentiation, and that additional BMP signals

were needed. Upon addition of these factors, increased generation of HPC was found. Also, the authors point at the importance of timing of addition of these factors. In a report by Wang *et al.* [72], different signaling inhibitors and activators were assessed for generation of $CD43^+$ HPC from hemogenic endothelium. Here it was shown, that inhibition of TGFβ signaling, activation of FGF signaling or retinoic acid signaling, greatly advances the generation of HPC.

It has to be noted that none of these reports assessed true stem cell properties and it remains questionable whether the HPC generated in this way will be useful as such. It is clear that addition of factors, which play an essential role during *in vivo* hematopoiesis, positively influence the generation of HPC from hESC.

Another option is the forced induction of HSC properties, by overexpression of factors which regulate them (figure 4), so called hematopoietic reprogramming. This has been successfully achieved by over-expression of HOXB4 in murine ESC-derived hematopoietic cells [39]. This method is based on the observation that HOXB4 expression enhances engraftment when overexpressed in bone marrow progenitors and the fact that this factor regulates HSC properties. Upon transduction of YS progenitors with ectopic HOXB4, it was shown that these cells also acquired HSC properties. Using an inducible expression system, the same HSC properties were conferred to ESC derived progenitors. This shows, that brief induction of HOXB4 is sufficient for conferring HSC properties. These cells were not only able to be successfully engraft, but also show multilineage commitment *in vivo*. Adaptation of this method to hESC did not confer similar properties to hESC-derived HPC [47].

Given the success of reprogramming somatic cells towards PSC [20], multiple research groups pursued along a similar line. Several attempts were made to directly reprogram cells towards HSC. This reprogramming can be initiated by directly inducing the desired cell characteristics, through overexpression of lineage specific genes. This has been successfully described for reprogramming of fibroblasts towards cardiomyocytes, which serves as a proof of concept [73]. In a report by Szabo et al [74], overexpression of OCT4 only reprograms fibroblasts to hematopoietic cells. These cells expressed the panhematopoietic marker CD45 and seemed to bypass the pluripotent state, as the obtained CD45 cells were shown to be unable to form teratomas *in vivo*. This proves that overexpression of OCT4 alone did not direct the fibroblasts towards a pluripotent state, but directly induced

hematopoietic properties. The authors were able to show that these cells could differentiate towards myeloid, erythroid and megakaryocytic lineages. However lymphoid differentiation was not addressed. Hematopoietic engraftment potential was found when injecting these cells into sublethally irradiated mice. As also evidenced in other reports generating $CD34^+$ cells from PSC, these cells were of myeloid lineage, arguing against true HSC properties of the injected cells. To obtain hemogenic endothelium, a more controlled hematopoietic reprogramming was described. Here, murine fibroblasts were reprogrammed to hemogenic endothelium using overexpression of four factors, namely *Gata2*, *Gfi1b*, *cFos* and *Etv6* [75].

Recently Doulatov *et al.* [76] have shown that overexpression of five hematopoietic factors effectively confers short term engraftment and expansion capacity to HPC generated *in vitro*. Here, HPC were derived from hESC, which were transduced with inducible vectors expressing *ERG*, *HOXA9* and *RORA*. Upon induction of these factors, a transcriptional program quite similar to that of HSC was activated. However, expression of these factors was insufficient for successful engraftment of these hESC-derived cells. Through reverse screening, the authors were able to determine that only cells that were dependent on two additional factors, *SOX4* and *MYB*, were capable of short-term engraftment.

Finally, Riddell et al [77] reported the feasibility of direct reprogramming of committed hematopoietic cells to HSC in the murine system (termed induced HSC or iHSC). After isolation of mature blood cells and transient expression of *Run1t1*, *Hlf*, *Lmo2*, *Prdm5*, *Pbx1*, *Zfp37*, *Meis1* and *Mycn*, HSC properties were conferred to these cells. These cells were able to reconstitute immune deficient mice. Upon secondary transfer, hematopoietic reconstitution was again observed.

Currently, the changes in genetic program underlying this reprograming are not well understood. However, this proof of concept shows the feasibility of reprograming approaches to HSC as well as PSC. Based on the cited reports, there is no consensus on the factors needed for successful hematopoietic reprogramming. Also, it might be that these factors differ between man and mouse.

8. Conclusion

Although major advances have been made towards generating gene corrected pluripotent stem cells for therapy, current protocols generating hematopoietic stem cells from these cells are lacking. Further understanding of the mechanisms underlying embryonic hematopoiesis will inevitably broaden our understanding of the *in vitro* differentiation processes and may result in efficient generation of HSC from PSC. Direct reprogramming of somatic cells such as fibroblasts towards hematopoietic stem cells using defined factors is currently an exciting alternative and a major advance in the field of *in vitro* hematopoiesis.

Conflict of interest:

The authors declare no conflict of interest.

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7. Discussion of the research in part I

The research described in part I aimed at assessing whether *bona fide* hematopoietic stem cells (HSC) are formed during *in vitro* hematopoiesis from human pluripotent stem cells (PSC). The formation of HSC *in vitro* could be of great applicability for the clinic, where compatible grafts are often limited or not available.

7.1 Can hematopoietic stem cells be generated from pluripotent stem cells?

During the last decade, different protocols have been described that reproducibly induce hematopoietic differentiation from PSC. However, it is unclear whether *bona fide* HSC are generated in these cultures

As described in the introduction, a *bona fide* HSC should fulfil three criteria: it should be multipotent, it should be able to self-renew and it should be able to engraft a sublethally irradiated host (upon secondary transplant).

Evidence is accumulating that all types of hematopoietic cells can be generated from lineage restricted multipotent progenitor cells which are derived from hemogenic endothelium, rather than from HSC: recent reports show that the embryo develops all blood cell lineages before the formation of a HSC during the definitive wave of YS hematopoiesis (1, 2). While erythro-myloid restricted precursors (EMP) and lympho-myeloid precursors (LMP) are lineage restricted precursors, cumulatively these precursors form all types of blood cells. EMP have been shown to give rise to erythroid cells, megakaryocytic cells, granulocytes and monocytic cells (such as Kupffer cells and microglia), while LMP give rise to myeloid cells and lymphoid cells, such as NK, T and B cells (1, 2).

Often CFU assays are used to assess the potential of hESC derived hematopoietic progenitors (3). From these data multipotent CFU-GEMM progenitors are estimated to constitute 0.4% of $CD34^+$ progenitors and 4% of $CD34^+CD43^+$ progenitors (4, 5). However, this assessment of multipotency does not include lymphoid differentiation

potential (T, NK, B cell precursor activity) and thus lacks proof for the presence of all blood lineages among the progeny of a single hematopoietic progenitor.

Taken together, multipotency, presumed by the generation of all types of blood cells is no formal proof for the occurrence of HSC.

Several research groups have taken the engraftment capacity of PSC-derived multipotent progenitors as proof for the presence of HSC (6-8). All these reports do show presence of human CD45 expressing hematopoietic cells in the blood or in the bone marrow of engrafted mice. However, no formal proof for the presence of a HSC that has all three aforementioned properties has been given.

In the study described in this thesis, an alternative approach was used to address whether HSC are formed during *in vitro* hESC differentiation and at the same time identify them. We reasoned that if HSC are formed in hESC culture, these cells should express high levels of MYB. MYB is expressed in fetal as well as postnatal HSC. In addition, MYB is absolutely required for HSC function as gene deletion mutant mice do not survive beyond E15, the point at which fetal hematopoiesis becomes HSC dependent (9, 10). This is in sharp contrast with YS-based hematopoiesis, which is MYB independent (1). We found that during *in vitro* hESC differentiation, no eGFP-marked MYB^+ cells are formed that are capable of multilineage differentiation. This suggests that hematopoiesis in culture is HSC independent (5). In addition, in these cultures macrophages are formed that closely resemble the tissue macrophages generated during YS hematopoiesis. Based on data from previous reports and our finding that no multipotent MYB^+ cells could be found, we concluded that *in vitro* hESC differentiation resembles YS HSC independent hematopoiesis.

In conclusion, it is clear that pluripotent stem cells are able to form every type of hematopoietic cell. If conditions and cues are right, HSC can be generated, as has been show by the generation of HSC in teratomas (11). However, the generation of HSC from pluripotent stem cells *in vitro* using current protocols has not been shown conclusively.

7.2 Can transgenic pluripotent stem cell lines be used for optimization of the in vitro generation of HSC?

Reporter cell lines, such as the MYB-eGFP hESC line we generated (5), can be used for the optimization of *in vitro* protocols to generate HSC. Use of such a reporter line provides a direct read-out for emergence of cells that express a key HSC marker. In combination with defined surface markers, this provides a powerful tool to screen for HSC formation.

In the murine system, the combination of reporter strains has led to the detailed description of several hematopoietic progenitor stages. One example is the combination of PU.1 and GATA1 reporters (12), which allowed for definition of precursors of common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). Combination of such lineage specific transgenic markers provides a tool to track progenitor commitment. An example of a reporter strain to identify and isolate HSC is the Fgd5 reporter, which was recently described (13). In the case of PSC differentiation, HSC specific markers could be combined with lineage specific markers. For example, the *MYB-eGFP* reporter we generated could be combined with an erythroid marker (e.g. GATA1 or a globin locus) and a myeloid marker (e.g. PU.1 or MPO). This would allow the definition of conditions that do not induce lineage skewing of the generated progenitors.

As tailored nuclease technologies were still rapidly evolving when we designed our study, we circumvented these technical issues by using random BAC integration.
The study was conceptualized in 2009-2010. At this time ZFN were a developing technology for genome editing, and reports showing successful endogenous integration of transgenes were still ample (14). Also, this technique was of low efficiency for many loci and flexibility in transgene design was low. Adding to this that homologous recombination in hESC remains cumbersome, we chose to use a random transgenic reporter method as was previously publishes in both the murine and human system. More flexible and efficient methods, such as the TALEN and CRISPR systems, only became available later on during the project (2012-2013) (15, 16).

The use of a BAC reporter, which is integrated *at random*, has been described and validated before, both in the murine and in the human system (17-20). It can be argued that random integration might influence faithful expression of the transgene (21). However, we found complete integration of the full BAC plasmid (200kbp) in the genome. This large integration should sufficiently isolate the MYB transgene from neighboring genomic sequences to ensure physiologic expression. eGFP expression was validated by showing a correlation between MYB mRNA levels and eGFP expression in multiple sorted populations.

Undoubtedly, the field will see the emergence of more transgenic PSC lines with the description and implementation of the new and highly efficient transgenic methods, such as CRISPR technology and it's derivatives.

7.3 Is there an advantage in using PSC derived cells to current gene therapy for hematologic diseases?

Currently, HSC transduced with retro- or lentiviral overexpression vectors are used for gene therapy for hematological diseases such as SCID-X1 and WAS (22-24). SCID-X1 patients carry a genetic defect in the interleukin 2 common gamma chain (IL2RG gene. These patients have low numbers of T and NK cells, as a defective common gamma chain leads to defective signaling for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Patients suffer from severe immunodeficiency with recurrent life threatening infection (25). In the case of WAS, a genetic defect in the WASp protein (Wiskott-Aldrich syndrome protein), causes defective acting polymerization. This is essential for T cell activation and effector functions. Like SCID-X1 patients, WAS patients also suffer from severe immunodeficiency (26). These genetic defects are thus ideal candidates for gene therapy, as introduction of a functional copy of the gene should alleviate disease symptoms. A major drawback of this approach is the risk of oncogenic transformation (27, 28). This is largely due to the insertional mutagenesis induced by transcriptional enhancers encoded within the viral sequences. These enhancers can induce aberrant expression of nearby genes, causing oncogenic transformation in the case of proto-oncogenes. These issues have been largely alleviated by use of later generation viral vectors, in which these enhancing

sequences become inactivated upon integration (29). However the use of promoters, which induce a constitutive increase in gene expression in HSC might still impose problems for the correction of proto-oncogenic genes.

Recently Genovese *et al.* reported a proof of principle for genetic engineering using tailored nucleases to correct a mutation in patient-derived HSC (30). The advantage of this method is that the corrected gene is inserted into its endogenous genomic locus. Expression of the corrected gene is thus dynamically regulated and expression levels are within a physiologically relevant range. The fact that no additional promoter sequences or viral sequences need to be inserted alleviates possible issues regarding insertional mutagenesis. While this is a promising advance for the field, this method holds some limitations: endogenous targeting of HSC is relatively inefficient and HSC in which off-target cutting or integration has occurred cannot be removed.

These limitations are circumvented when autologous iPSC are used for targeting instead of HSC.

Corrected iPSC can be cultured indefinitely without differentiation. This not only has the advantage that an infinite amount of cells could be provided for transplantation, it also allows for thorough screening of the manipulated iPSC. Using iPSC, cells can be screened and expanded in a clonal manner. In this way, cells in which off-target cutting of the nucleases, off-target integration of the donor cassette or in which no correction has occurred can be excluded. The corrected pluripotent stem cells can be stored in iPSC banks. This might render the search for suitable donors obsolete. Generation of patient specific iPSC, their genetic correction and the generation of sufficient number of cells for transplantation requires valuable time and efforts. Herefore use of histocompatible iPSC banks might prove a more feasible alternative and provide a rapidly accessible source of gene corrected PSC.

For the generation of HSC from PSC, one option would be to further optimize *in vitro* PSC differentiation protocols. Study of the ontogeny of the hematopoietic system has already identified several essential factors for generation of HSC. Among these are BMP-signaling (31, 32), Wnt-signaling (33) and Notch signaling (34, 35). The effect of these factors has been tested in hESC differentiation cultures without significant improvements to the generation of HSC in these cultures (36-38). However, the signals that regulate hematopoiesis might prove to be more complex than initially

thought, as essential non-hematopoietic cell types which act on the developing hematopoietic system are located outside of the site of hematopoiesis or are needed before the onset of hematopoiesis. Such an example was provided in the murine system, where catecholamines produced by the sympathetic nervous system influence HSC generation in the AGM region (39). In the zebrafish model, Wnt signaling early in ontogeny was found essential for expression of notch ligands by the somites. This signaling was shown to be essential for the specification of hemogenic endothelium in the AGM region (40).

To increase the efficiency of hESC derived definitive hematopoiesis, efforts have been undertaken to block the primitive hematopoietic wave during hESC differentiation using chemical compounds. By blocking activin-nodal signaling, which promotes Wnt signaling, the efficiency of definitive type hematopoietic cell generation improves. This increase in definitive type hematopoietic progenitors improved the efficiency of T cell generation in these cultures (41).

Even when improved culture methods become available which favor HSC generation from PSC, maintenance of these cells *in vitro* might prove problematic resulting in rapid differentiation of the cells. Currently no culture conditions are described which can sustain and expand HSC properties. Al of the described HSC culture conditions induce rapid differentiation of LT-HSC (42-44). It has to be noted that the field has made advances in retaining HSC properties upon short-term culture by blocking the mTor pathway and stimulating the Wnt pathway. These HSC are cultured in absence of cytokines, which might induce differentiation (45). The question is whether such an approach could be integrated with differentiation of pluripotent stem cells, which depends on instruction of cytokines for generation of hematopoietic cells.

Thus in conclusion, while the generation of cells for gene therapy using PSC remains promising, the generation of bona fide transplantable HSC has not been described. For this reason, the current applicability of PSC derived cells remains limited to cases where long lived hematopoietic cells can be transplanted (T cells or macrophages, in the case of SCID-X and Hurler syndrome respectively). As methods for the *in vitro* differentiation of PSC are increasing in efficiency, this might provide feasible for clinical application.

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Part II:

In vitro generation of innate lymphoid cells from hematopoietic stem cells

1. Summary of part II

The immune system is composed of two components, the innate and adaptive immune system. The innate immune system provides a rapid, broadly effective response to infection, while the adaptive immune system is highly specific and effective in clearing infection later on. Both systems rely on the function of specific types of immune cells.

While the first member of the innate lymphoid cell (ILC) family, the natural killer cell (NK cell) was described 30 years ago, several previously unidentified members were recently added to this family. ILC have important roles in both health and disease. NK and ILC1 aid in the immune defense against viruses and malignant cells. Other types of ILC are essential to develop specific lymphoid organs and maintain gut homeostasis, lymphoid tissue inducer cells (LTi) and ILC3 respectively. Another type of ILC has been described to be involved in the development of asthma and defense against parasites (ILC2).

As most members of the ILC family have only been described during the last decade, the development of these cells from HSC is not studied in detail. Progress in understanding the nature and development of ILC has been made in the murine system. Here, key transcription factors, environmental cues involved in ILC development, and the lineage relationships between different types of ILC have been largely, although not completely, elucidated. Some reports have described plasticity of ILC subsets during their development, as certain types of ILC were described to give rise to others. This suggests a flexible model, where ILC can change their function upon environmental cues. However, other reports have argued against such a model, and were not able to find this plasticity.

In the human system, development of different ILC remains elusive, and reports generally rely on *ex vivo* culture of distinct developmental stages found in secondary lymphoid tissues (SLT). Using such a system, plasticity of ILC3 and NK cells was described. Here ILC3 were able to develop into NK cells, upon loss of stimulation of the aryl hydrocarbon receptor (AHR). Another report, which used an *in vitro* differentiation system of cord blood $CD34^+$ progenitors argues against this plasticity, as ILC3 were not found to generate NK cells in absence of strong AHR stimulation.

As the developmental stages of ILC3 have not been elucidated in the human system, this might simply reflect the differences in plasticity of different stages of ILC3 development.

In our study, we set out to define the developmental stages through which NK and ILC3 progress. We found that as in SLT, stage 3 NK precursors could be detected, defined by a $CD34^{-}CD94^{-}CD117^{+}$ phenotype. We describe that within this stage 3 population, ILC3 can be distinguished from true stage 3 precursors, as ILC3 display a $CD117^{hi}IL1R1^{+}NKp44^{+}$ phenotype, while stage 3 precursors are $CD117^{dim}NKp44^{-}$. Within the $CD117^{dim}$ population, both $IL1R1^{-}$ and $IL1R1^{+}$ precursors could be found. These $IL1R1^{-}$ stage 3 precursors seem primed towards the NK lineage, as assessed by the expression of high levels of Tbet and absence of RORγt, while $IL1R1^{+}$ stage 3 precursors show ILC3 lineage priming evidenced by downregulation of Tbet and upregulation of RORγt. This priming is also reflected in the lineage potential of these cells. Although the $IL1R1^{-}$ stage 3 population gave rise to some $IL1R1^{+}$ stage 3 cells and ILC3, these cells rapidly and predominantly generated NK cells. On the other hand $IL1R1^{+}$ stage 3 cells rapidly developed into ILC3 cells, but retained NK developmental potential. Upon addition of FICZ, a strong AHR agonist, development of NK cells $IL1R1^{+}$ stage 3 cells was hampered, and development of ILC3 promoted. The progeny of $IL1R1^{-}$ stage 3 cells was largely the same in the presence or absence of FICZ.

These data thus show, that early in development, ILC3 precursors retain the potential to develop into NK cells and *vice versa*. Here, strong signaling through the AHR is essential for ILC3 to develop further. This finding might thus unify the views on ILC3 lineage plasticity.

2. Samenvatting van deel II

Het immuunsysteem bestaat uit twee componenten, het aangeboren en het verworven immuunsysteem. Het aangeboren immuunsysteem zorgt voor een snelle respons op infectie, met een brede werking. Het verworven immuunsysteem daarentegen is hoog specifiek en klaart de infectie efficiënt, hetzij met enige vertraging. Beide systemen zijn gebaseerd op de functie van specifieke soorten immuuncellen.

De eerste soort aangeboren lymfoïde cel (*innate lymphoid cell*, ILC), de *natural killer cell* (NK cel) werd 30 jaar geleden beschreven. Recent werden verschillende soorten ILC aan deze familie toegevoegd, die voorheen niet beschreven waren. De classificatie van ILC binnen het aangeboren en verworven immuunsysteem is niet zo eenvoudig, en ILC worden vaak beschreven als de brug tussen beide componenten van het immuunsysteem. ILC hebben een belangrijke rol in verschillende ziektebeelden, maar zijn ook essentieel in het behoud van immunologisch evenwicht in afwezigheid van infectie. NK cellen en ILC1 hebben een belangrijke rol in de immuun respons tegen virussen en belemmert kwaadaardige ontwikkeling van cellen. Andere types ILC zijn belangrijk voor de ontwikkeling van bepaalde lymfoïde organen en voor het behoud van het immunologisch evenwicht in de darm, deze worden respectievelijk *lymphoid tissue inducer cells* (LTi) en ILC3 genoemd. Een ander type ILC met een rol in de ontwikkeling van ,onder andere, astma werd beschreven (ILC2).

Gezien de meeste leden van de ILC familie tijdens het laatste decennium werden beschreven zijn er nog vele vragen omtrent hun ontwikkeling. Door middel van onderzoek in het muismodel werden reeds inzichten verkregen in de eigenschappen van ILC en hun ontwikkeling. Zo werden de transcriptie factoren, omgevingsfactoren nodig voor ILC ontwikkeling en de verwantschappen tussen verschillende soorten ILC grotendeels, maar niet volledig, beschreven. Sommige artikels beschrijven het bestaan van plasticiteit van verschillende types ILC tijdens hun ontwikkeling, gezien uit sommige ILC andere soorten kunnen worden gegenereerd. Dit is suggestief voor een flexibel model, waarin ILC hun functie kunnen veranderen onder invloed van omgevingsfactoren. Andere publicaties beweren echter het tegendeel, en konden deze plasticiteit niet aantonen.

Bij de mens is de ontwikkeling van ILC nog niet volledig in kaart gebracht, en de artikels die deze ontwikkeling beschrijven zijn gebaseerd op de kweek van ILC types buiten het lichaam. Meestal worden bepaalde ontwikkelingsstadia uit secundaire lymfoïde organen (secondary lymphoid tisses, SLT) geïsoleerd en in kweek gebracht. Door middel van dergelijk model, kon plasticiteit tussen ILC3 en NK cellen worden aangetoond. ILC3 waren in staat om NK cellen te vormen, als stimulatie van de aryl hydrocarbon receptor (AHR) wegviel. In een andere publicatie die gebruik maakte van een *in vitro* differentiatiesysteem van $CD34^+$ precursoren geïsoleerd uit navelstrengbloed kon deze plasticiteit niet worden aangetoond. Gezien de verschillende stappen in de ontwikkeling van ILC3 nog niet beschreven zijn bij de mens, is het mogelijk dat deze verschillen in plasticiteit te wijten zijn aan verschillen in de differentiatie van deze cellen.

Onze studie had als doel de stappen in de ontwikkeling van NK en ILC3 te beschrijven. We vonden dat stadium 3 NK cel precursoren aanwezig waren in deze kweken, net als in SLT. Deze konden geïdentificeerd worden op basis van de expressie van volgend oppervlaktemerker profiel: $CD34^-CD94^-CD117^+$. We beschreven dat binnen deze stadium 3 populatie, een onderscheid kan worden gemaakt tussen ILC3 en echte stadium 3 precursoren, gezien ILC3 een $CD117^{sterk}IL1R1^+NKp44^+$ oppervlaktemerker profiel vertonen. Binnen de $CD117^{zwak}$ populatie konden zowel precursoren met een $IL1R1^-NKp44^-$ als een $IL1R1^+NKp44^-$ fenotype worden gedetecteerd. De $IL1R1^-$ stadium 3 precursoren leken eerder aan te leunen bij NK cellen, gezien deze Tbet sterk tot expressie brengen, maar geen RORγt expressie vertonen. Anderzijds verlagen $IL1R1^-$ stadium 3 precursoren hun expressie van Tbet, maar verhogen de expressie van RORγt. Het aanleunen bij een bepaald celtype kon ook worden aangetoond door de analyse van de dochtercellen van deze precursoren. Ook al waren $IL1R1^-$ stadium 3 precursoren beperkt in staat om $IL1R1^+$ stadium 3 precursoren te vormen en later ILC3, toch vormden deze precursoren vooral snel NK cellen. Anderzijds ontwikkelen $IL1R1^-$ stadium 3 precursoren zich snel tot ILC3, maar behielden toch het potentieel om NK cellen te vormen. Na toevoeging van FICZ, een sterke agonist van de AHR, werd de ontwikkeling van NK cellen uit $IL1R1^+$stadium 3 precursoren tegengewerkt en werd de ontwikkeling van ILC3 bevorderd. De dochtercellen van de $IL1R1^-$ stadium 3 precursoren waren gelijkaardig in de aan- of afwezigheid van FICZ.

Deze data tonen dus aan dat ILC3 precursoren hun potentieel behouden om NK cellen te vormen tijdens vroege ontwikkeling, en omgekeerd. Tijdens deze ontwikkeling is sterke stimulatie van de AHR noodzakelijk voor de verdere ontwikkeling van ILC3. Deze bevinding kan mogelijk de verschillende visies op ILC plasticiteit samenbrengen.

3. Introduction of part II

3.1 The immune system

The human body is constantly engulfed by a wide variety of commensal and pathogenic bacteria, fungi and viruses. However, infection is a rare event. Pathogens are prevented from entering the body by potent physical, microbial and chemical barriers which are under control of the immune system. When the pathogen succeeds in crossing these barriers, infection is usually rapidly cleared by the immune system and does not lead to disease.

When pathogens enter the body, tissue resident macrophages come in to action. The first cells that fight the infection will engulf the invading pathogens. These cells will then rapidly attract different types of more specific immune cells. Through secretion of cytokines and chemokines, this leads to a process called inflammation. The first cells that arrive at the site of infection are cells of the innate immune system, while the second wave consists of cells of the adaptive immune system.

3.2 The innate immune system

The innate immune system is evolutionarily conserved and is found in both vertebrates and invertebrates. The innate immune system has the ability to rapidly, selectively and efficiently target invading pathogens through the action of a humoral and cellular component. This causes a first line of defense during the first hours/days after infection.

One of the modes of action of the innate immune system, is activation of the complement system. The complement system is part of the humoral innate immunity and consists of multiple complement factors circulating in the blood in an inactive state. The enzymatic function of the complement factors is activated upon their cleavage at the site of infection. Here complement factors elicit one of three major actions: 1) they bind to the pathogen and trigger their engulfment by phagocytic cells,

2) they act as chemo-attractants for immune cells such as neutrophils and monocytes and 3) they create pores in bacterial cells, leading to their direct killing (1).

The cellular part of the innate immune system is activated upon recognition of pathogens in several manners. One of these is the recognition of pathogen-associated molecular patterns (PAMP's). PAMP's are molecular structures, which are conserved on a wide range of different pathogens. Examples of PAMP's are lipopolysaccharides of gram-negative bacteria and lipotechoic acid of gram-positive bacteria (2). These structures are essential for the function of pathogens, and are thus not prone to mutational diversity. Targeting of such conserved sequences prevents the occurrence of mutants that can escape the immune system. It also allows the innate immune system to be highly effective against a wide range of pathogens, by expression of germline-encoded receptors. These germline-encoded receptors are called pattern recognition receptors (PRR)(3). PAMP's trigger PRR on the tissue resident immune cells and on cells that form the barrier of the body, such as epithelia. One example of such a receptor is the TOLL-like receptor expressed on macrophages and neutrophils, which will lead to activation of the NFkB pathways upon triggering (2). Activation of macrophages and neutrophils, leads to the production of chemokines and cytokines that promote the differentiation and expansion of immature innate immune cells. Also, additional innate immune cells are activated and attracted through this mode of action.

The cellular innate immune system is composed of several cellular components. These include macrophages, mast cells, dendritic cells, various granulocytes and natural killer (NK) lymphocytes (figure 1).

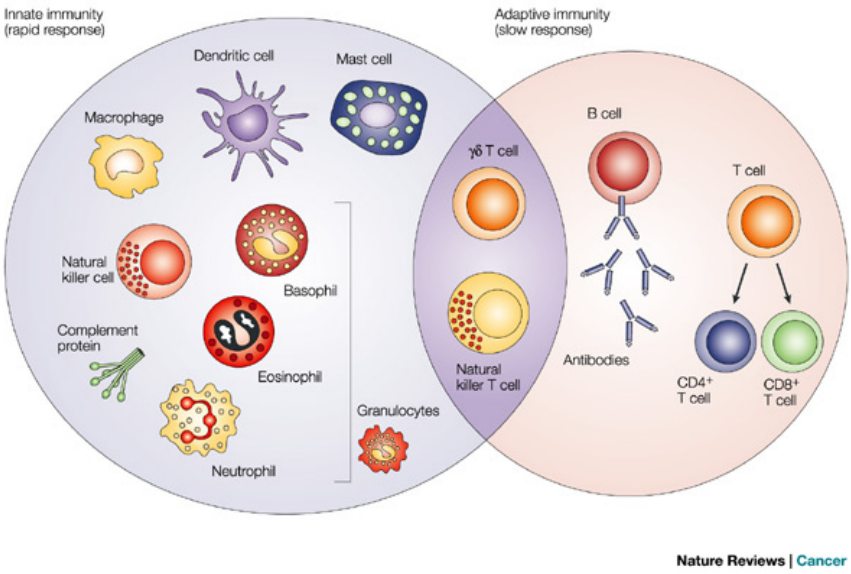

Figure 1: *Key components of the immune system.*

The immune system consist of two components, the innate and adaptive immune system. The innate immune system provides a rapid response to infection, through the action of complement factors, granulocytes, natural killer cells, macrophages and mast cells. The adaptive immune system functions through the action of B and T cells, which carry highly specific immune receptors. (Figure adapted from Dranoff G et al. (4))

The first cells that fight infection are macrophages, which are attracted to the site of infection and eliminate pathogens or damaged cells by phagocytosis. The ingested particle is encompassed into a *phagosome*. Once inside the cell, the phagosome will fuse with a lysosome and form the phagolysosome. The factors contained therein, such as hydrogen peroxide and hydroxyl radicals will kill the contained pathogen or cell (2). Another innate cell type, the dendritic cell, arises from the same precursor as the macrophage. The naïve dendritic cells take up pathogens through engulfment. The DC process the ingested proteins into peptides, which are then presented on the major histocompatibility complex (MHC) to the T lymphocytes of the adaptive immune system.

While macrophages are often the first to detect the presence of pathogens, the readily attract other types of immune cells. Granulocytes, such as neutrophils, are attracted to the site of infection and will extravasate from the blood stream into the

tissues. Alternatively blood borne pathogens can trigger endothelial cells to recruit neutrophils to the site of infection. Neutrophils play an important role in clearing infections. These can directly kill bacteria and fungi by release of their toxic granular content (myeloperoxidase and lysozyme), in a process called degranulation and take up the encountered pathogens by phagocytosis. Phagocytocis is mediated through recognition of complement or recognition of antibody through FcγR. Patients having mutations in any of these pathways fail to efficiently clear infection (reviewed by Witko-Sarsat (5)). In addition, neutrophils also interact with several parts of the adaptive immune system (reviewed by Nauseef et al (6)).

Natural killer (NK) cells do not directly target the invading pathogens, but rather target cells of the body that are infected with intracellular bacteria and viruses or which have undergone oncogenic transformation. NK cells express an array of activating and inhibitory receptors, and the balance of the signals through these receptors triggers killing of the infected cell. Among these activating receptors are the C-type lectin receptors CD94-NKG2C, NKG2D, NKG2E/H and NKG2F, the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, and the killer cell immunoglobulin-like receptors (KIRs) KIR-2DS and KIR-3DS and FcRγIIIa (CD16). Inhibiting receptors consist of CD94-NKG2A/B heterodimers and KIR-2DL and KIR-3DL (7, 8). One of the major mechanisms of NK cell triggering is recognition of non-self (Figure 2). NK cells receive signals through their activating receptors, priming them to kill infected cells. However they also constantly “scan” for the level of MHCI molecules expressed on the surface of the cells of the body. Healthy cells express high levels of MHCI, which causes triggering of the KIRs on the NK cells, and subsequently inhibits killing of the healthy cell. When MHCI levels drop, which is frequently induced by viral infection, the strength of these inhibitory signals drops and triggers the NK cell to kill the infected cell (1, 9). This process is called “missing-self recognition” (figure 2). Another method of action is the process of “non-self-recognition”. Here the NK cell recognizes molecules that are not expressed by host, but are encoded by the infecting pathogen. Alternatively, pathogens or malignant transformation might cause upregulation of the expression of self-molecules, which are otherwise not expressed, or can only be detected at low levels. This leads to the process of “stress-induced self-recognition” (figure 2) (7-9).

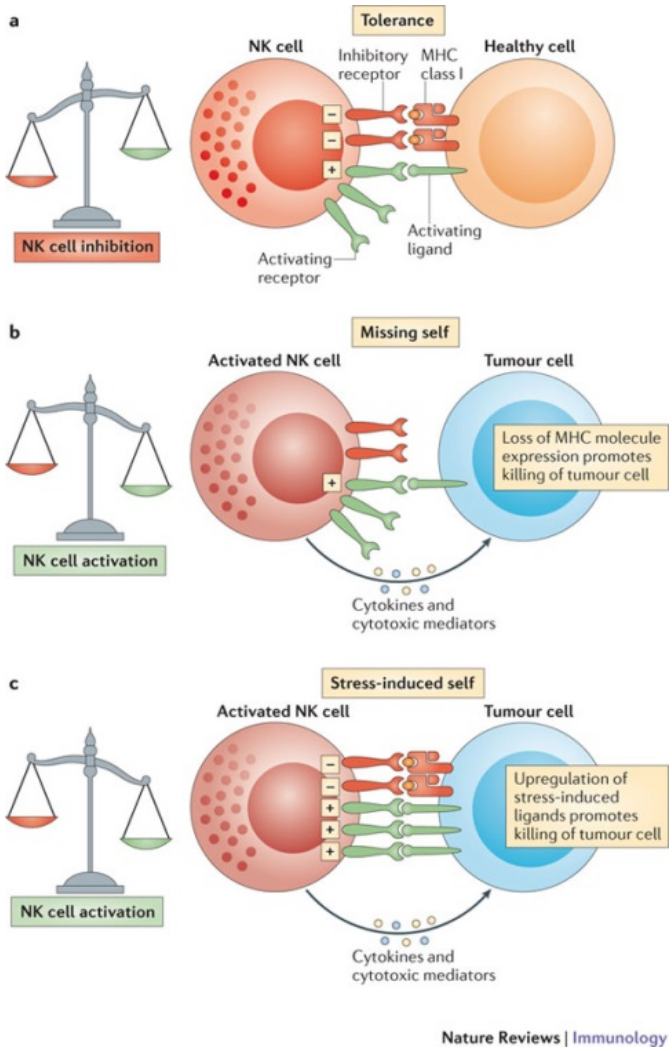

Figure 2: *Methods of natural killer (NK) cell activation.*

Activation of NK cells is regulated by the balance of signaling through inhibitory and activating receptors. If inhibitory signals (e.g. recognition of self through MHCI detection) exceed the strength of activating signals, NK cell function is inhibited. If upon infection, inhibitory signals cease, or if activating signals increase, NK cells become activated (Figure adapted from Vivier, E et al. (9)).

Together, these mechanisms effectively fight bacterial or viral infection during the first hours and days after infection. In cases where the infection is not successfully cleared, it is usually sufficiently contained until the adaptive immune system clears the infection.

3.3 The adaptive immune system

The adaptive immune system is a slow but very powerful system to fight and clear infection. The adaptive immune system has evolved during vertebrate evolution. In this system, each cell has a unique receptor. The specificity of these is generated through rearrangement of the germ line DNA encoding immune receptors. This occurs by the merit of “recombination activating” enzymes (RAG) mediate in rearrangement of the genetic sequence, as described further. The immune receptor that is the result of this rearrangement is different for each cell, which leads to an immense diversity within the pool of immune cells. These cells are collectively able to recognize a wide variety of pathogens. Different types of adaptive immune cells come into action upon infection: B cells provide immunity through antibody production and T cells by direct killing of infected cells or by regulation of immune responses by other immune cells.

When a cell with a specific immune receptor recognizes a pathogen, it expands in a clonal manner. This cellular proliferation takes days or even weeks. The adaptive immune response is therefore inherently slow to develop, but, once the cells are generated, provides a more efficient mechanism than the innate immune system, and is able to clear infection. In addition, cells which have been successful in recognizing and eliminating pathogens or infected cells will survive and generate an immunological ”memory”, that can rapidly come in to action upon re-infection.

3.3.1 T cell immunity

T cells are characterized by the expression of a hetero-dimeric T cell receptor (TCR). T cells are subdivided into cells that express a TCRαβ heterodimeric receptor or a TCRγδ heterodimeric receptor.

T cells also express co-receptors, which aid binding of the TCR to the MHC complex. Two types of co-receptors exist, namely CD4 or CD8 co-receptor molecules. TCRαβ

T-cells are subdivided into helper T-cells ($CD4^+$), cytotoxic T-cells ($CD8^+$) and regulatory T cells.

$CD4^+$ $TCR\alpha\beta^+$ cells will help to activate other immune cells, such as B cells, $CD8^+$ effector T cells and macrophages, while $CD8^+$ $TCR\alpha\beta^+$ effector cells will directly kill infected cells. Regulatory T cells (Treg) are a subtype of $CD4^+$ T cells that control autoimmunity, through limitation of the activity of immune cells after they have exerted their function. Treg have also been described to play a role in preventing allergy and asthma. On the other hand, Treg also exert negative effects, as they suppress immune responses against different cancers (10-12). They are generally characterized as $CD4^+$ cells expressing high levels of CD25 and the Forkhead transcription factor Foxp3 (13).

After clonal expansion and the elimination of infection, T cells go into apoptosis (contraction) and only a pool of memory T cells survive, which can be rapidly activated an expanded upon re-infection. T cell memory is subdivided into central memory (Tcm) and effector memory (Tem), while the first migrate to secondary lymphoid tissues (SLT) and are expanded upon antigen triggering, the latter readily go to infected tissues to exert their effector function (14).

Cells carrying a TCRγδ are mainly found in mucosal tissues, for example as intra epithelial lymphocytes (IEL) in the gut, where they take part in the first line of defense against invading pathogens (15, 16).

Although the precursor that generates T cells originates in the bone marrow (17), T cells develop in a specialized organ, called the thymus. Herein all necessary cues, such as Notch signaling and interleukin-7 (IL-7), are provided for commitment to the T cell lineage and subsequent development (reviewed by Taghon and Rothenberg (18)). After selection on the basis of their functionality, cells exit the thymus and home to the secondary lymphoid tissues.

Development of T cells is characterized on the molecular level by rearrangement of the T cell receptor genes and expression of surface molecules such as CD4 and CD8. Generally, T cell development is divided into consecutive stages, during which the developing T cell undergoes three essential processes. These will be described in the following paragraphs: 1) β -selection, 2) positive selection and 3) negative selection.

T cells develop through several steps defined by the expression of their co-receptors and T cell receptor (figure 3). During the double negative (DN) stage cells are defined by the lack of expression of a TCR and are negative for CD4 and CD8. At this stage, the T cell precursor will either further differentiate along the TCRαβ or the TCRγδ lineage. After this stage, TCRαβ differentiating cells enter the immature single positive stage (ISP), which is $CD4^+CD8^-$. During the ISP stage, the selection of a successfully rearranged β-TCR chain occurs through the process of β-selection. After successful progression through this stage, cells upregulate the CD8 molecule and become double positive (DP). In the end, a DP T cell will develop into either a single positive $CD4^+$ T cell (T helper cell) or $CD8^+$ T cell (cytotoxic T cell)(reviewed by Singer *et al.* (19)).

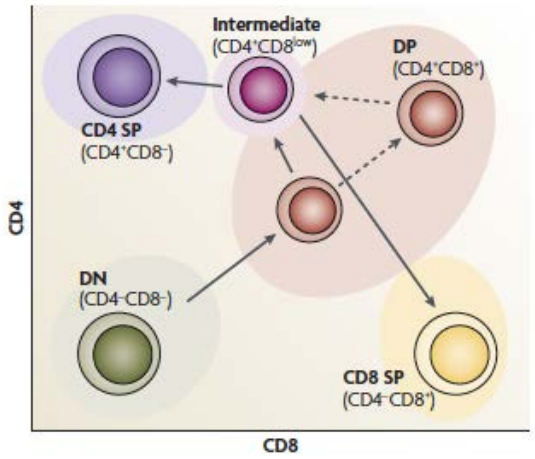

Figure 3: *Stages in T cell development.*
During the development of T cells, a $CD4^-CD8^-$ (double negative, DN) precursor, will upregulate CD4 and CD8 expression, to become a $CD4^+CD8^+$ (double positive, DP) precursor. This DP precursor will either develop into a CD4 single positive T cell or a CD8 single positive T cell (Figure adapted from Singer et al. (19)).

Each T cell expresses a unique T cell receptor, and only recognizes one specific antigen. However, together the pool of these T cell receptors recognizes an extensive array of different antigens. Through this mechanism, they form an effective means to clear infection by different pathogens. This clonal specificity is achieved by rearrangement of the T cell receptor gene loci during their development. The TCR are encoded by 3 TCR loci, which consist of different gene segments. The gene segments consist of multiple variable (V), diversity (D), domain (J) gene segments

encoding the variable domains and one or two gene segments encoding the the constant domain (C). The V-D-J segments are “recombined” by two enzymes called “recombination activating genes” RAG1 and RAG2. These interact with one-another and “cut-and-paste” V-J or V-D-J segments in a random fashion. This generates a random diversity in antigen recognition. Adding another layer to the diversity, endo- and exonucleases remove or add several nucleotides between these VDJ gene segments. This process generates unique T cell receptors which recognize one single antigen (1).

The process of recombination is a process of trial and error and cells with unsuccessful TCR-locus rearrangement will fail to develop further. During development, the cell is submitted to several quality control mechanisms. One of the quality control steps during αβ T cell development is control of successful β chain rearrangement, in a process called β-selection. During β-selection a cell that has successfully rearranged the TCRB encoding genomic locus will dimerize with a pre-Tα chain, a germline encoded surrogate for the TCRα chain, to form a pre-TCR. If the pre-TCR is formed, cells will be selected for further development.

Once the TCRα chain is rearranged, the generated TCR will be screened for weak interaction with self-MHC complexes, which is essential for immune function. Through this process, double positive cells will develop into either a MHCI restricted ($CD8^+$ single positive T cells) or MHCII restricted ($CD4^+$ single positive T cells).

After β-selection and positive selection, the T cells generated are subjected to one final test before it exiting the thymus: negative selection. This mechanism excludes cells that recognize self-antigens, which would otherwise lead to autoimmunity.

3.3.1.1 T helper responses.

$CD4^+$ T cells have a role in promoting antibody production and induction of $CD8^+$ cytotoxic T cell responses. These $CD4^+$ cells are subdivided into different helper

subsets, based on the cytokines they produce and the immune response they elicit (figure 4).

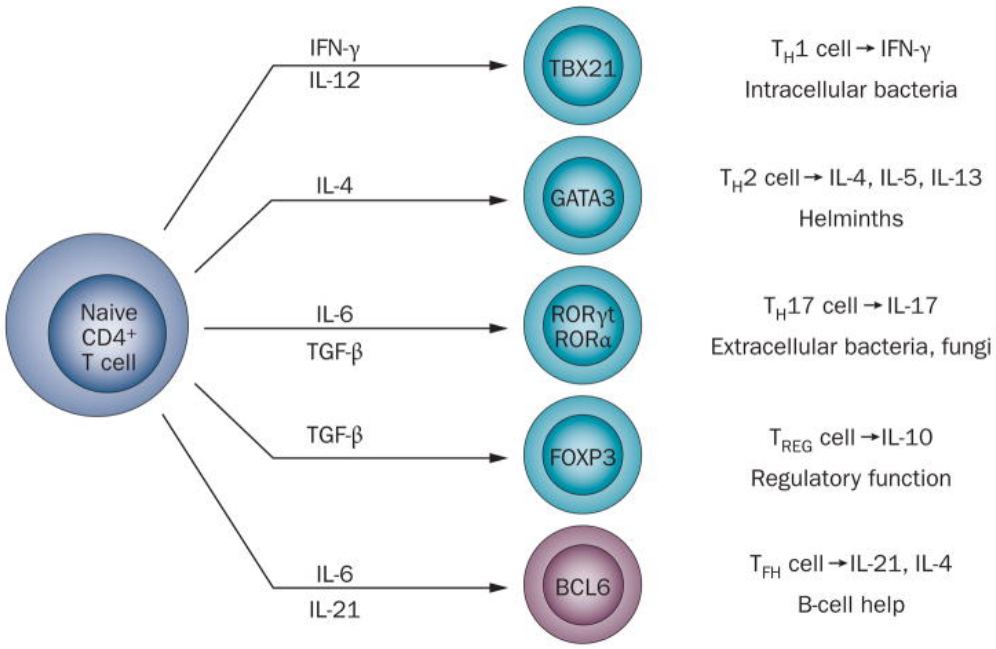

Figure 4: *Different types of T helper responses.*
T helper responses are divided into different subtypes: the TH1, TH2 TH17 and T_{FH} response. During a TH1 response, type I cytokines are produces, such as interferon gamma (IFN-γ) and IL-2. The TH2 response on the other hand leads to production of type2 cytokines such as IL-4, IL-5 and IL-13. The Th17 response leads to production of TNF, IL-17 and IL-22, while T follicular helper (T_{FH}) responses lead to IL-4 and IL-21 production (Figure adapted from Craft et al. (20))

Classically, T helper subsets are subdivided into T helper 1 (TH1) and T helper 2 (TH2) responses. TH1 cells produce type1 cytokines such as interleukin 2 (IL-2), IL-3, interferon gamma (IFN-γ), and granulocyte-macrophage colony stimulating factor (GM-CSF). TH2 cells produce IL-3, IL-4 and IL-5. TH1 and TH2 responses protect against intracellular pathogens and extracellular pathogens, respectively. Another T helper subset, the TH17, produces TNF, IL-17 and IL-22. TH17 cells provide protection against bacteria and fungi. The T_{FH} response, produced IL-4 and IL-21 provides survival and differentiation signals for B cells (20).

3.4 Bridging innate and adaptive immunity – innate lymphoid cells

Various cell types have been described that bridge innate and adaptive immunity. Among these are γδ T cells, natural killer T cells (NKT) and B1 B cells. While these have re-arranged immune receptors, except for γδ T cells, these receptors display limited diversity (21, 22). These cell types play an important role in the rapid responses to particular pathogens. Another type of lymphoid cells that bridges the innate and adaptive immune system are closely related to NK cells, but have effector functions similar to T cell subsets (figure 5). These are collectively termed "innate lymphoid cells" or ILC. During recent years this "innate counterpart" of T-helper subsets has been the focus of intense investigation (23).

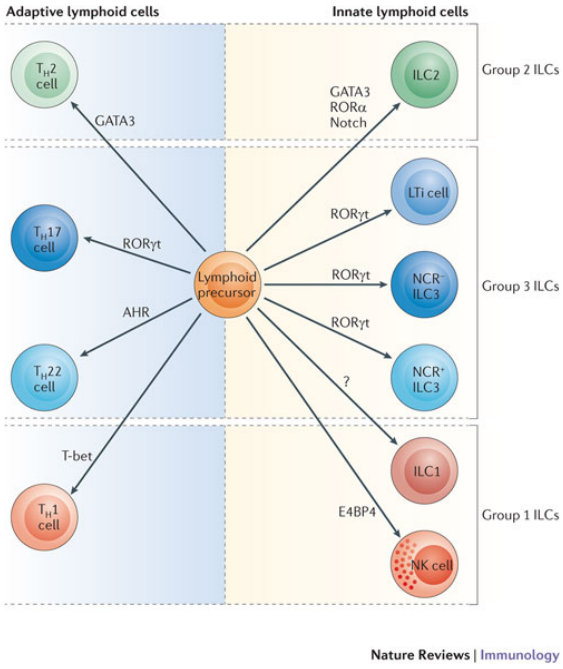

Figure 5: *Analogy between adaptive T helper subsets and innate lymphoid cell subsets.*

Innate lymphoid cells (ILC) have been divided in three separate subsets based on their cytokine production profile. Group 1 ILC contain natural killer (NK) and ILC1 cells, group 2 ILC contain ILC2 and group 3 ILC contain lymphoid tissue inducer cells (LTi) and natural cytotoxicity receptor (NCR) positive and negative ILC3 (Figure adapted from Walker et al. (24)).

ILC secrete immunoregulatory cytokines, similar to their T-counterparts. The main difference between innate lymphoid cells (ILC) and T cells is the absence of rearranged T cell receptor, ILC produce cytokines in an antigen receptor independent manner. Different types of ILC have been described to have a function in mucosal immunity, repair of damaged tissues and the generation of lymphoid organs.

3.4.1 Types and characteristics of innate lymphoid cells

As several research groups described ILC's simultaneously, these have been given a myriad of names. Recently a more uniform nomenclature has been proposed by Hergen Spits and James Di Santo (23). Here, for clarity, only this nomenclature will be used.

Using this classification, the family of ILC consists of different subsets defined by their cytokine secretion profile in analogy with T-helper subsets. These three subsets are called group 1 ILC, group 2 ILC and group 3 ILC (24) (table I and figure 6 and 7).

In brief, group 1 ILC contain conventional NK cells and type 1 ILC (ILC1) Group 2 ILC contain type2 ILC (ILC2) while group 3 ILC are constituted of type3 ILC (ILC3) and lymphoid tissue inducer (LTi) cells.

TableI: different subsets of innate lymphoid cells and their cytokine production profile (based on Spits and Di Santo (23)).

Class	**Cell type**	**Cytokines produced**
Group 1 ILC	NK	TNF-α, IFN-γ, perforin, granzyme
	ILC1	IFN-γ
Group 2 ILC	ILC2	IL-5, IL-9, IL-13
Group 3 ILC	NCR+ ILC3	IL-22
	NCR- ILC3	IL-17, IL-22
	LTi	IL-17, IL-22

3.4.1.1 Group 1 ILC

- NK cells have an important function in killing infected or transformed cells. NK cells exert their function through release of perforin and granzymes. NK cells also have immunoregulatory functions by the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) (25).

- ILC1 cells have been described to produce IFN-γ, but lack cytotoxic activity. Based on their capacity to secrete type1 cytokines, these cells were classified as group1 ILC However it is still not clear to which lineage these cells belong: as progeny of cells generated from ILC3, as an induced functional state of ILC3 or as a sub-class of NK cells, as will be described lower in section 3.4.3.2 (26).

3.4.1.2 Group 2 ILC

ILC2 cells were first described in the murine system to fight Helminth infection in absence of B and T cells (27). Human ILC2 have also been described since (28). These cells produce IL-5, IL-9 and IL-13, similar to T helper 2 cells (28). ILC2 cells are found in tonsils, blood and intestines, where their main function is the elimination of extracellular parasites.

3.4.1.3 Group 3 ILC

- LTi cells are essential for the development of secondary lymphoid tissues such as lymph nodes and Peyers patches. These cells lack expression of the natural cytotoxicity receptors (NCR) NKp46 and NKp44 (29) (see tableII). LTi have a major role during fetal development, but have also been described in secondary lymphoid tissues after birth (30).

- ILC3 express NCR (NKp44 and NKp46), but lack expression of other NK cell markers, such as KIRs, CD16 and CD94 (31). They do not produce TNF-α and IFN-γ, but are a main source of IL-17 and IL-22. ILC3 are found mainly in the small and large intestine, where they provide mucosal immunity and form a barrier against invading pathogens (32).

While it is becoming clear that ILC2 arise through a different developmental pathway than ILC1 and ILC3 subsets. The relationships between group 1 and group 3 ILC, or different group 3 ILC subtypes remain a matter of debate. It is possible that these different subsets represent the same cell type that produces different cytokines or expresses different surface markers depending on different environmental cues.

3.4.2 Phenotype of innate lymphoid cells

A range of surface markers have been described which could enable isolation of different types of ILC and discrimination from other lymphoid cell types (table II). NK cells are generally defined by expression of CD94, natural cytotoxicity receptors (NCR) such as NKp44 and NKp46 and positivity for CD56. Group 1 ILC can be discriminated from other types of ILC, by the absence of IL1R1 expression. ILC1, in contrast to NK cells do not express NCR. Group 3 ILC are also positive for IL23R, CD117 and some subtypes express NCR. Among these group 3 ILC, ILC3 can be defined by expression of CD56, NKp44 and NKp46, while LTi are negative for the latter two. Group 2 ILC can be discriminated by expression of CRTH2, a marker uniquely expressed on this subset.

Table II: Phenotype of innate lymphoid cells (taken from Spits et al. Nature Reviews Immunology, 2013 (23)).

Marker	Group 1 ILCs		Group 2 ILCs	Group 3 ILCs	
	NK cells	ILC1s	ILC2s	LTi cells	NCR^+ ILC3s*
CD4	−	−	−	−	−
CD25	−/+‡	low	low	ND	low
CD56	+	−	ND	−	50%
CD117 (also known as KIT)	−	−	+/−	+	+
CD127 (also known as IL-7Rα)	−/+§	+	+	+	+
CD161	−/+§	+/−	+	+/−	+
NKp44 (also known as NCR2)	−/+‖	−	−	−	+
ICOS	low	+	+	ND	+
NKp46 (also known as NCR1)	+	−	−	−	+
CRTH2	−	−	+	−	−
IL-1R	−	+	+	+	+
IL-23R	−	−	ND	+	+
IL-12Rβ2	+	+	−	−	−
ST2 (a subunit of IL-33R)	−	−	+	−	−
IL-17RB (a subunit of IL-25R)	−	−	+	−	−
CRTH2, chemoattractant receptor-homologous molecule expressed on T_H2 cells; ICOS, inducible T cell co-stimulator; IL, interleukin; ILC, innate lymphoid cell; LTi, lymphoid tissue-inducer; NCR, natural cytotoxicity triggering receptor; ND, not determined; NK, natural killer.					
*Also referred to as NK22 cells.					
‡$CD56^{hi}CD16^-$ cells express CD25.					
§Not on all cells.					
‖NKp44 is expressed by activated but not resting NK cells.					

3.4.3 Development of innate lymphoid cells

Innate lymphoid cells develop from a common lymphoid progenitor (CLP), which is dependent on the transcriptional repressor Id2 and will develop into a presumptive common ILC precursor. Induction of Id2 is crucial for this differentiation as it inhibits the function of E proteins. This is important to block T- and B cell development from this common precursor, as both T- and B cells rely on function of E proteins for their commitment (33). From this common precursor, differentiation into the different types of innate lymphoid cells depends on the expression of specific transcription factors. These are in common with the factors regulating differentiation of their adaptive counterparts (figure 5 and figure 6).

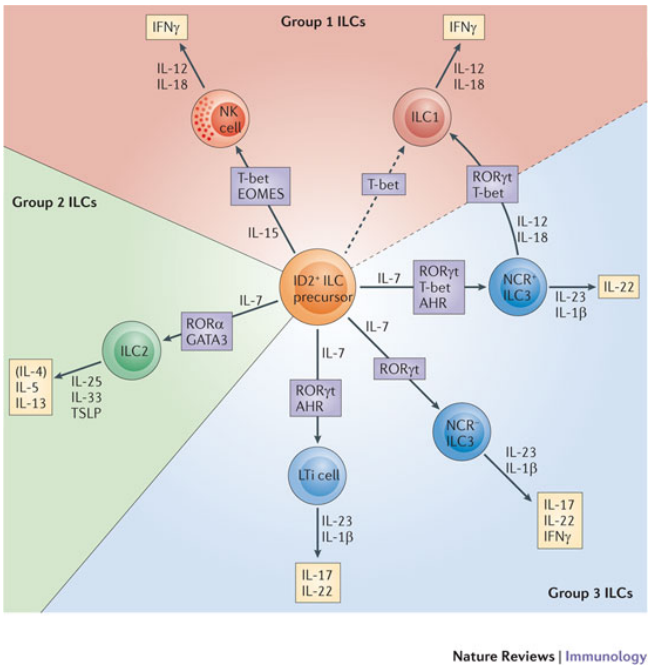

Figure 6: *Innate lymphoid cell subsets and the queues regulating their development.*

Different groups of ILC develop from a common ID2$^+$ precursor through induction of different transcription factors. Group 1 ILC develop under the influence of IL-15 signaling (NK cells) and IL-7 (ILC1), during their development T-bet and Eomes are key transcription factors. Group 2 ILC develop through induction of the transcription factors RORα and GATA3, and depend on IL-7 and Notch signaling. Group 3 on the other hand strictly depend on RORγt signaling for their development, and develop in the presence IL-7 and aryl hydrocarbon receptor (AHR) signaling (Figure adapted from Spits et al. (23)).

3.4.3.1 *Development of conventional NK cells*

The development of human conventional NK cells has been divided into 5 different stages and has been largely based on the work of the group of Michael Caligiuri.

It has been established that NK cells develop from a precursor common to T and B cells. This common lymphoid precursor (CLP) is derived from bone marrow HSC. The human CLP has been defined as a $CD34^{+}CD38^{+}CD45RA^{+}CD10^{+}$ cell (figure 7) and can be detected both in the BM and the SLT (34). The CLP then differentiates into a stage 2 NK cell precursor, which is marked by the loss of CD10 expression and upregulation of CD117 expression. This cell then differentiates into a stage 3 precursor, which loses expression of CD34. This stage 3 precursor is highly heterogenous, containing precursors of NK cells and innate lymphoid cells. The bulk of stage 3 precursors is minimally defined by a $CD94^{-}CD117^{+}$ surface phenotype. From this stage 3 precursor, conventional NK cells will develop, and upregulate CD94 expression (stage 4 and stage 5). Stage 4 NK cells are defined by a $CD94^{hi}CD56^{bright}$ phenotype and have a more cytokine producing profile. From this stage 4 NK cell, although still under debate, stage 5 NK cells will develop, which have a more cytotoxic profile (35-37).

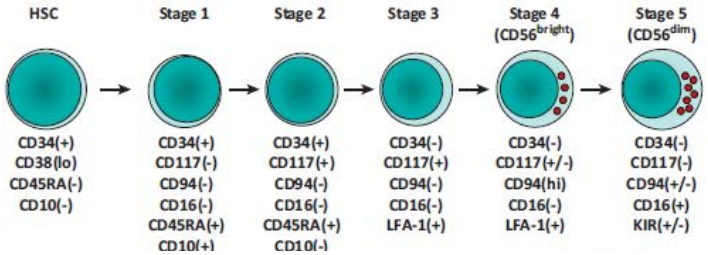

***Figure 7**: A schematic overview of human NK cell development.*

NK cell development has been divided into separate stages based on surface markers and functionality. From a hematopoietic stem cell (HSC), NK cells develop towards through several progenitor stages (stage 1-3) to become $CD56^{bright}CD94^{hi}$ stage 4 NK cells, which have a cytokine producing profile. These can then develop into stage 5 $CD56^{dim}CD94^{low}$ NK cells, which have a cytotoxic profile (Figure adapted from Freud et al. (38)).

3.4.3.2 Lineage relationships between innate lymphoid cells

Whether NK cells and other types of ILC derive from a common ILC precursor or whether they diverge at the CLP stage remains a matter of scientific debate (figure 8).

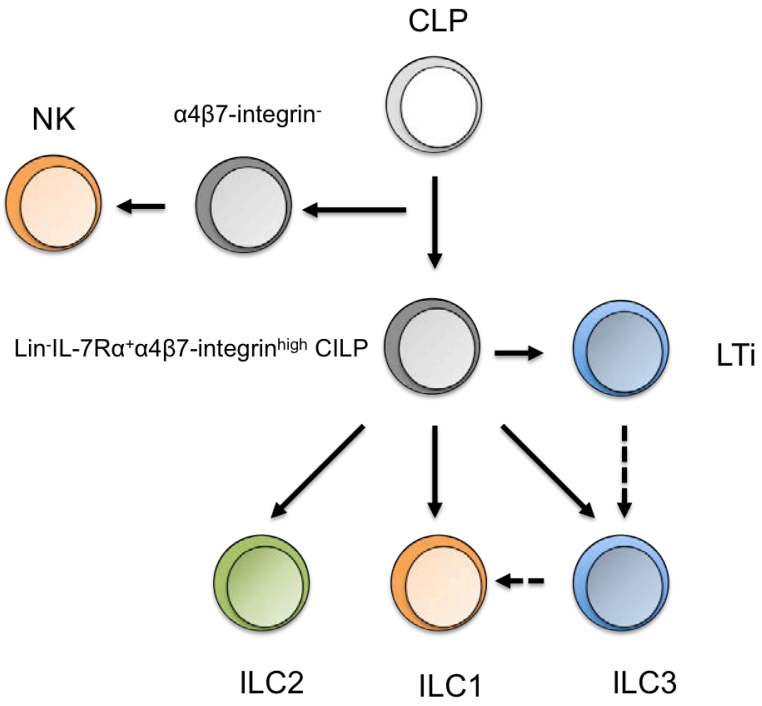

***Figure 8**: a schematic overview of innate lymphoid cell development.*

All ILC develop from the common lymphoid progenitor (CLP). From this stage different environmental cues divert cells into the ILC lineage, and block T and B cell differentiation. The exact lineage relationships between these different subsets and the stages through which they develop, have not been described in detail. Whether ILC1 are a subset of NK cells, or develop from ILC3 remains topic of debate.

In the murine system it was shown that all ILC, except NK cells, derive from a common precursor (39). This common precursor was defined within the Lin$^-$IL-7Rα$^+$α4β7-integrin^{high} population, and was found to express high levels of the transcription factor PLZF (39). This was strengthened by the publication by Klose *et al.* (40), where a common precursor for all ILC types, except NK cells was described. It has been proposed that NK and ILC3 have a different origin using adoptive transfer experiments and tracing of the lineage of RORγt expressing precursors (a key

transcription factor during type 3 ILC development) (41, 42). In a report by Crellin *et al.* (31), a different precursor for NK and ILC was proposed on the basis of expression of CD127 (IL-7 receptor) and both were detected in human SLT. Here the $CD127^+$ precursor gave rise to ILC3 cells, while the $CD127^-$ precursor gave rise to NK cells. This adds to the evidence for a separate precursor of ILC3 and NK.

Whether there is plasticity between ILC3 and NK cell subsets remains a matter of debate. Using cord blood $CD34^+$ *in vitro* cultures, human NK and ILC3 cells were described to be fully committed cells without plasticity (43). In contrast, plasticity of SLT derived NK cells and type 3 IL-22 producing ILC was described *ex vivo* (34, 44).

A different origin of $ROR\gamma t^+$ LTi and $ROR\gamma t^+$ ILC3 has been described based on expression of α4β7-integrin. Sawa and colleagues have shown that in fetal liver $ROR\gamma t^+$ α4β7-$integrin^+$ precursors give rise to LTi, while their α4β7 integrin negative counterpart gives rise to ILC3 (45). On the other hand it was shown that $NKp46^-$ ILC (presumptive LTi-like cells) can give rise to $NKp46^+$ ILC (ILC3), which would argue for a common origin of both cell types (42). Also in the human system, *ex vivo* $NKp44^-$ $CD117^+$ LTi were shown to be able to differentiate into $NKp44^+$ ILC3 when stimulated with IL-2, IL-23 and IL-1β in culture (26).

This thus warrants further research to define the exact lineage relationships of ILC subtypes and the different stages in their respective development.

3.4.3.3 Transcriptional control of innate lymphoid cells

Group 1 ILC develop by induction of the transcription factor T-bet, while NK cell development diverges from the CLP by up regulation of EOMES induced by IL-15 signaling (46, 47). NK cells are also dependent on E4BP4/Nfil3 and TOX for their differentiation. While these genes were thought to uniquely drive NK cell development, recently Seillet *et al.* and Geiger *et al.* provided evidence for an important role of E4BP4/Nfil3 in ILC2, LTi and ILC3 development (48, 49). Also, TOX was shown to play a role in LTi development, next to its role in NK development (50). Developmental plasticity has been described between group 3 ILC and ILC1 when

cultured in presence of IL-12 and IL-18 , which questions ILC1 to be a distinct cell type. These data argue for a common origin of both cell types or alternatively might reflect alternative activation stages of a highly plastic common ILC. Therefore, environmental cues may downregulate RORγt in response to IL-12 and IL-23 (42). However, whether ILC1 and ILC3 are truly the same cell type remains a question and requires further research.

ILC2 have been described to depend on a different set of transcription factors, which are thought to be unique for ILC2 during development of innate lymphoid cells: RORα (51) and GATA3 (52). Again, this remains subject of debate, as GATA3 signaling has also been described to be important for ILC3 development (53).

In conclusion, while key transcription factors regulating development of innate lymphoid cell subsets have been defined, the exact relationships between these subsets, their plasticity, and the interplay between factors involved has not been fully clarified.

3.4.3.4 Extracellular signals regulate innate lymphoid cell development

The cues that direct the development of ILC are being unraveled. NK cells were shown the be dependent on IL-15 signaling for their development, while all other types of ILC rely on IL-7 signaling (41, 43). Essential for ILC3 development is signaling through the AHR. Genetic knockout of this receptor leads to abolishment of ILC3 expansion and failure to clear *Citrobactor rodentium* infection in the murine model (54, 55). In a recent report by Hughes *et al* (56), it was shown that stimulation of AHR blocked differentiation of human secondary lymphoid tissue derived ILC3 towards NK cells. Similarly, IL-1β signaling has also been described to block NK cell development from these ILC3 (57).

Within group 3 ILC, Notch signaling has been described to be important for postnatal ILC3 development, while LTi development has been described to be Notch independent (58). Similarly, ILC2 are dependent on Notch signaling for their development (59). ILC2 cells are closely related to T cells, as thymic DN1 and DN2 precursors can still develop into ILC2 cells. The key factor blocking T cell

development at this point has again been shown to be Id2 (60). Here, the strength of Notch signaling seems to influence the choice between T and ILC2 lineage development from a common thymic progenitor, where weak notch signals drives these precursor towards the T lineage and strong Notch signals give rise to ILC2 cells (59).

3.4.4 Function of innate lymphoid cells

In contrast to NK cells, which constitute approximately 15% of peripheral blood lymphocytes, other types of ILC are generally not detected in the circulation (0.01-0.1% of circulating cells) (61). These are found to be tissue resident and reside in tonsils, thymus, gut and skin (62).

While the key role of NK cells in the immune system has been established, the roles of other types of ILC are only just being elucidated.

3.4.4.1 Type 1 ILC

The function of type 1 ILC is similar to that of TH1 T cells, and these produce type 1 cytokines such as IFNγ and TNF. NK cells have an important role in the immunological defense against viral infections (63). They also have a protective role against cancer development (64). NK cells act through several mechanisms to exert their effects. MHCI expression is reduced upon malignant transformation, to evade the action of T lymphocytes. However, this causes NK cells to elicit a missing-self response and attack the malignant cells. Alternatively, several oncogenic malignancies have been described to upregulate molecules that trigger NK cell activation through NKG2D, even on cells with normal MHCI levels (65). Some cancers also express ligands for the NKp30 activating receptor (66).

While evidence is limited, ILC1 have been described to have a protective role against extracellular parasites (40). It has also been shown that a specified ILC1 in the human system, which has no characteristics of ILC3 or NK cells, is able to produce

IFNγ upon bacterial infection of the gastro-intestinal tract. A rapid antibacterial response is hereby provided (26).

In the case of inflammatory bowel disease, an increased number of ILC1 cells were found in the intestine of patients with Crohn's disease (67). These ILC1 can then produce IFNγ in a TH1-like response, causing increased inflammation of the intestine.

3.4.4.2 Type 2 ILC

Type 2 ILC have been described to have a protective role against gastrointestinal parasites such as Helminths through the production of IL-25 by epithelial cells. IL-25 will trigger ILC2 to produce IL-5 and IL-13, eliciting TH2 immune responses and attraction of eosinophils (68).

In the case of asthma, PRR expressed on airway epithelial cells are triggered by PAMPs, leading to production of cytokines, chemokines and antimicrobial peptides. This causes attraction of immune cells, of which monocytes play an important role. These monocytes will differentiate into dendritic cells, which will activate specific TH2 T cells in the lymph nodes (69).
Among the cytokines produced after endothelial cell PRR triggering, are IL-25 and IL-33. These latter two cytokines trigger ILC2 to produce IL-5 and IL-13. IL-5 will lead to the attraction of eosinophils, which cause basement membrane deposition (70). One characteristic of asthma is an increase in goblet cells (goblet cell metaplasia or GCM), which is induced by IL-13 signaling on endothelial cells (71).

3.4.4.3 Type 3 ILC

While ILC mainly have immune protective functions, some reports have described a cancer-promoting role of ILC3 through an IL23 induced mechanism. Herein cancer development is promoted by production of IL-17 and IL-22 (72). Chronic inflammation of the intestine has been shown to lead to colorectal cancer, through downregulation

of IL-22 binding protein (IL22BP) production. IL22BP neutralizes soluble IL22, and thus maintains a subtle balance of IL-22 levels in homeostasis (73).

ILC3 have been described to play and essential role in infections of the gastro-intestinal tract. In mouse models infection with Citrobacter rodentium leads to upregulation of IL-22 by ILC3 and mice lacking ILC3 die because of this bacterial infection (74). IL-22 stimulates the gastro-intestinal epithelial cells to produce anti-microbial peptides and defensins, which will clear bacterial infection (75).

As innate lymphoid cells play a role in intestinal mucosa homeostasis, it is maybe not surprising that a role for ILC has been described in intestinal bowel disease (IBD). TH17 responses have been described to play a role in sustaining inflammatory bowel disease (76, 77), thus a similar role for ILC3 has been proposed. Indeed, NCR^- ILC3 have been found in increased levels in the colon of patients with Crohn's disease (78). IL-22, produced by ILC3, can trigger signaling on epithelial cells, which causes STAT3-related signaling. This has important anti-inflammatory roles and causes restitution of goblet cells in colitis (79).

In addition, innate lymphoid cells have been described to play a role in the development of inflammatory immune diseases such as psoriasis (80) and asthma (81). Psoriasis is caused by deregulated immune responses of the skin. Here skin-resident dendritic cells produce IL-12 and IL-23. This leads to a pro-inflammatory TH1 and TH17 response, which induces production of IFNγ, TNFα, IL-17 and IL-22. These cytokines promote inflammation and increase keratinocyte proliferation. Interestingly, while IL-23 has been described to promote growth and activation of ILC3, IL-12 has been described to alter ILC3 development towards ILC1 type cells (26), which produce IL-17 and IL-22 and IFNγ and TNFα respectively.

IL-22 producing innate lymphoid cells have been described to play an essential role in protecting against graft-versus-host disease (GVHD) related intestinal inflammatory damage. Frequency of ILC and the levels of IL-22 were both reported to be lower upon GVHD (43).

It was recently described that innate lymphoid cells interact with cells of the adaptive immune system. Next to their role as cytokine producers, ILC3 have been described to present peptides in MHCII molecules (29, 82, 83). This might provide a novel

mechanism of antigen presentation, but understanding this specific function requires further research.

In conclusion, future research will certainly unravel more immune functions of ILC and elucidate their role in both protective immunity and in disease. Finally, this could lead to therapies directly targeting innate lymphoid cells.

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5. Research focus of part II

Innate lymphoid cells (ILC) have been recently discovered as the innate counterpart of T helper adaptive lymphoid subsets. These cells were described to have an essential role in maintaining mucosal homeostasis and play important roles in stimulating the immune system upon infection. Understanding of the differentiation of immune cells, the transcription factors and extracellular signals involved, is essential in understanding the immune system in both health and disease.

The exact developmental intermediates through which ILC differentiate remain a matter of scientific debate and it remains to be established whether conventional NK cells and other types of ILC originate from a common precursor, and whether there is lineage plasticity between these lineages.

The *in vitro* differentiation of cord blood progenitors towards ILC enables analysis of stepwise development through different intermediates. Thorough understanding of ILC development will provide the field with insights into pathogenesis of ILC related diseases and might provide novel therapeutic targets. The efficient generation of different types of ILC *in vitro* can also provide cells which are suitable for transplantation.

We thus set out to define the lineage relationships between conventional NK cells and group 3 ILC using this *in vitro* study. As plasticity has been described and refuted between these ILC subtypes, we hypothesized that this could be due to differences in the lineage commitment during differentiation of these cells. We thus assessed the earliest progenitors of the cells which arose *in vitro* for their lineage potential, and defined whether AHR signaling is necessary at any one of these stages and is able to influence *in vitro* ILC3 versus NK cell commitment.

In the presented work, we describe the stepwise differentiation of progenitors *in vitro* similar to the stages described in *ex vivo* studies. We show that during this differentiation both $IL1R1^+$ and $IL1R1^-$ stage 3 NK precursor can be detected. These either develop into a $IL1R1^+$ ILC biased precursor or retains its phenotype to become an NK primed precursor. This $IL1R1^+$ stage 3 cell presents itself as a bipotent conventional NK and ILC3 precursor. Signaling through the AHR receptor can

enforce commitment towards ILC3 cells. We found that the IL1R1^{-} stage 3 NK committed precursor cannot be diverged from the NK developmental pathway.

The research presented here unifies different models of NK and ILC development, and presents the field with a novel tool for study of ILC and NK development, which allows for stepwise analysis of the genes and cues involved in the development of these cell types.

6. Publications

6.1 Lineage primed early stage 3 NK precursors with can be distinguished by the expression IL1R1, and have a differential dependency on AHR stimulation.

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Lineage primed early stage 3 NK precursors with can be distinguished by the expression IL1R1, and have a differential dependency on AHR stimulation .

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Abstract

During recent years, the phenotype and function of innate lymphoid cells has been unraveled both in the murine as in the human system. However, the exact developmental pathways of NK cells and ILC3 remain elusive. Here, using *in vitro* differentiation of human cord blood $CD34^+$ cells, we describe how the earliest NK and ILC3 precursors diverge at the stage 3 NK developmental stage. The stage 3 population contains both $IL1R1^-$ and $IL1R1^+$ precursors. The $IL1R1^+$ fraction is strongly $ROR\gamma t^+$ but displays some remnant Tbet expression, in contrast to fully mature ILC3 cells. The $IL1R1^-$ fraction is $ROR\gamma t^-$, and contains cells which express T-bet and low levels of Eomes. These $IL1R1^-$ cells partially remain IL1R1 negative and become NK cell primed precursors, while the remaining part gives rise to an $IL1R1^+$ stage 3 intermediate. This $IL1R1^+$ stage 3 intermediate either upregulates CD94, to generate NK cells, or further upregulates CD117 and NKp44, to become $ROR\gamma t^+$ ILC3. Addition of AHR agonist to these cultures diverts differentiation of the $IL1R1^+$ stage 3 precursor toward ILC3 development and hampers development of NK cells In contrast, differentiation of the $IL1R1^-$ stage 3 precursor towards the NK lineage was not influenced by AHR signaling. These data provide a novel insight in the early developmental decisions during the development of innate lymphoid cells and NK cells.

Introduction

The family of innate lymphoid cells (ILC) bridges innate and adaptive immunity. In contrast to immune cells of the adaptive immune system, ILC lack expression of a rearranged immune receptors, such as the T cell receptor (TCR) or B cell receptor (BCR). ILC produce immunoregulatory cytokines that act on mucosa and other types of immune cells. Different subsets of ILC have been described according to their respective cytokine production profiles. This divides the ILC family in three major groups: group 1, group 2 and group 3 ILC (1, 2). Group 1 ILC contain conventional NK cells (NK) and ILC1, which are both IFN-γ producing cells. Group 2 ILC contain only one member, ILC2, which produces IL-5, IL-9 and IL-13. Group 3 ILC contain IL-22 and IL-17 producing cells and is constituted of lymphoid tissue inducer cells (LTi) and ILC3.

ILC develop from a common lymphoid progenitor (CLP), which is dependent on Id2. Id2 function is key for differentiation of ILC at this stage as it inhibits the function of E proteins. This is important to block B cell and T cell potential from the CLP (3). The human bone marrow CLP has been defined by the $CD34^{+}CD38^{+}CD45RA^{+}CD10^{+}$ surface marker profile. Upon induction of an NK developmental program, the CLP differentiates into a stage 2 NK cell precursor and is marked by the loss of CD10 expression and upregulation of CD117 expression. These stage 2 NK cells subsequently differentiate into a stage 3 precursor, which loses expression of CD34. This population of stage 3 precursors is minimally defined by a $CD34^{-}CD94^{-}CD117^{+}$ surface phenotype and is highly heterogeneous and contains precursors of both NK cells and ILC. From this stage 3 precursor, conventional NK cells will develop, and upregulate CD94 expression to generate stage 4 and stage 5 NK cells. Stage 4 NK cells are defined by a $CD94^{+}CD56^{bright}$ phenotype. These cells have low cytotoxic capacity and mainly produce cytokines. From this stage 4 NK cell, although still under debate, stage 5 $CD94^{+}CD56^{dim}$ NK cells may develop, which have a more cytotoxic profile (4).

How different ILC populations develop and how their development fits in the aforementioned 5-stage NK cell development model remains elusive. In the human system, Hughes *et al.* described RORγt^{+} ILC3 isolated from adult secondary lymphoid tissues (SLT) to hold lineage plasticity and to be able to differentiate towards IFNγ producing NK cells (5). These ILC3 were described to express high levels of IL1R1 and it was shown that signaling through the aryl hydrocarbon receptor (AHR) was essential to inhibit their differentiation towards NK cells. In contrast, in a report by Ahn *et al.* it was shown that NK cells and ILC3, generated using a protocol based on cord blood CD34 differentiation *in vitro,* are irreversibly committed and do not show any lineage plasticity (6). These NK cells were defined by expression of LFA1, while IL-22 producing ILC3 do not express LFA1. The LFA1^{-} ILC3 were shown to be uniformly positive for IL1R1, while the LFA1^{+} NK cell precursors are devoid of IL1R1 expression. These data are also supported by data in the murine system, where using RORγt lineage tracing, NK cells derived from a RORγt^{+} precursor were not detected (7).

We hypothesized that these seemingly contradictive reports might reflect differences in the differentiation state of these populations. During early lineage development, cells which are not yet fully committed might be present and represent cells with higher plasticity. In this study we aimed at defining the developmental relationship of NK cells and ILC3 from the earliest stage 3 precursors arising during in vitro NK differentiation culture. As ILC3 development is clearly marked by the upregulation of IL1R1, while NK cells remain negative for IL1R1, we addressed whether differences in expression level at the stage 3 NK precursor population might reflect early skewing of these stage 3 precursors. The obtained data provide evidence for bifurcation of ILC development within the stage 3 precursor population, which is marked by IL1R1 expression. At this stage, the IL1R1^{-} precursor is unresponsive to AHR stimulation. On the other hand, AHR stimulation of the IL1R1^{+} stage 3 precursor provides a rapid progress into the ILC3 lineage, while hampering development towards the NK cell lineage. These data provide a novel insight in the early divergence of the developmental pathways of ILC3 and NK cells.

Materials and Methods

Isolation of cord blood $CD34^+$ precursors.

All cord blood samples were obtained from the Cord Blood Bank of the University Hospital Ghent following approval by the ethical committee of the Ghent University hospital. Cord blood samples were processed by mononuclear cell isolation, through ficoll gradient centrifugation (Lymphoprep, Axis-Shield, Oslo, Norway). Subsequently $CD34^+$ precursors were isolated using magnetic activated cell sorting (MACS) (Miltenyi biotec, Bergisch Gladbach, Germany) according to the manufacturers protocol. MACS CD34 isolation obtained purity >60% on average. To isolate pure precursors, cells were FACS sorted for following phenotype $CD34^+CD45^+CD14^-CD3^-CD19^-CD56^-$ ($CD34^+lin^-$) obtaining purities >98%.

Lymphoid differentiation of $CD34^+$ precursors.

$CD34^+$ precursors were differentiated *in vitro* as previously described (8, 9). In brief, 350 $CD34^+lin^-$ precursors were seeded on mitotically inactive EL08-1D12 feeders (a kind gift of professor E. Dzierzak (10)) seeded to confluence. Cells were cultured for up to 28 days in DMEM:F12 (2:1) (Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with 20% human serum (Innovative research, Novi, MI, USA), 20 ng/ml Stem Cell Factor (SCF) (Peprotech, Rocky Hill, NJ, USA), 5 ng/ml interleukin 3 (IL-3) (R&D Technologies, Minneapolis, MN, USA), 20 ng/ml IL-7 (R&D Technologies), 10ng/ml IL-15 (Peprotech), 10 ng/ml Flt3-L (R&D Technologies), 24µM β-mercaptoethanol, 50µM ethanolamine, 20 µg/ml ascorbic acid, 50 ng/ml sodium selenite, 1% penicillin+streptomycin, 1% pyruvate and 1% glutamin. Half of the medium was changed with fresh medium containing cytokines every 7 days and IL-3 was omitted after the first week of culture.

$CD34^+Lin^-$ FACS sorted populations were cultured using this protocol and were seeded as indicated. For AHR stimulation experiments, cultures were supplemented with either 300 nM 6-Formylindolo(3,2-b)carbazole (FICZ) (Enzo Life sciences, Farmingdale, NY, USA) or a similar volume of DMSO carrier.

Flow cytometric analysis and cell sorting

Cells were analyzed using a LSRII flow cytometer (BD, Franklin Lakes, NJ, USA) or FACS sorted using a FACS ARIA IIIu with ACDU unit. Samples were stained using the following antibodies: IL1R1 PE (R&D Technologies), IL1R1 APC (R&D technologies), NKp44 APC (eBioscience, San Diego, CA, USA), CD94 PerCP-Cy5.5 (BD biosciences, San Jose, CA, USA), CD117 PE-Cy7 (eBioscience), CD45 APC-Vio770 (Miltenyi biotec), CD56 V450 (BD bioscience).

For intracellular staining, cells were fixed and permeabilized after surface staining using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Following antibodies were used for transcription factor staining: RORγt-PE, Tbet-PE and Eomes-PE (all from eBioscience).

Real time RT-PCR

Cells were lysed using Qiazol lysis reagent (Qiagen, Venlo, Netherlands). RNA was extracted using the miRneasy RNeasy micro kit (Qiagen) and cDNA was synthesized using superscriptIII reverse transcriptase using random hexamer primers (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR reagents and SYBR GreenI were obtained from Roche (Roche, Penzberg, Germany) and used according to the manufacturer's instructions. The reactions were run on a lightcycler480, 384 well system (Roche). Following primers were used in this study: GAPDH fwd: 5'- TGCACCACCAACTGCTTAGC -3'; GAPDH rev: 5'- GGCATGGACTGTGGTCATGAG -3';

YWHAZ fwd: 5'- ACTTTTGGTACATTGTGGCTTCAA -3'; YWHAZ rev: 5'- CCGCCAGGACAAACCAGTAT – 3';

RORC fwd: 5'- TCGCCAAAGCATCCTGGCAAAG -3'; RORC rev: 5'- ATGGGGTGGAGGTGCTGGAAGA -3';

Tbet fwd: 5'- CGCCAGGAAGTTTCATTTGGG -3'; Tbet rev: 5'- TGGAGGGACTGGAGCACAAT -3';

Eomes fwd: 5'- AGCCCTCAAAGACCCAGACTT -3'; Eomes rev: 5'- CCAGGGACAATCTGATGGGAT -3'.

Statistical analysis

All statistical analyses were performed using SPSS V22.0 (IBM, New York, NY, USA). Significance was assessed using the students t test with significance level set at $p \leq 0.05$.

Results

ILC3 and conventional NK cells develop *in vitro* through a stage 3 NK cell precursor.

To analyze the kinetics of human innate lymphoid cell development, $CD34^{+}Lin^{-}$ cord blood precursors were cultured for up to 28 days in conditions which favor NK/ILC development. After 10 days of development, the majority of cells had acquired a $CD94^{-}CD117^{dim}$ phenotype and had downregulated CD34, in agreement with the presence of a stage 3 NK cell phenotype (Figure 1A and data not shown). After 14 days of culture, the first $CD94^{+}$ NK cells appeared. Simultaneously, $CD94^{-}CD117^{hi}$ cells were generated. Within the stage 3 population, part of the cells upregulated IL1R1, but remained negative for NKp44 expression. At the following time points, both the $CD94^{+}$ NK cell population and $CD94^{-}CD117^{hi}$ population vastly increased. By the end of culture, the $CD94^{+}$ NK cells were uniformly positive for CD56, but only a small fraction expressed NKp44 and IL1R1. In contrast, after 28 days of culture the $CD94^{-}CD117^{hi}$ population, uniformly expressed high levels of CD56, NKp44 and IL1R1. This latter surface phenotype is compatible with presence of ILC3 (Figure 1A). These $CD94^{-}CD117^{hi}$ cells expressed high levels of RORγt, but were negative for T-box transcription factors Tbet and Eomes. In contrast, the $CD94^{+}$ NK cell population shows weak expression of RORγt and expressed high levels of Tbet and Eomes (Figure 1B).

Thus three clear populations could be discerned during innate lymphoid cell differentiation: a $CD94^{+}$ NK cell population, and within the $CD94^{-}CD117^{+}$ population a $CD94^{-}CD117^{hi}IL1R1^{+}NKp44^{+}$ ILC3 population and a $CD94^{-}CD117^{dim}$ stage 3 population.

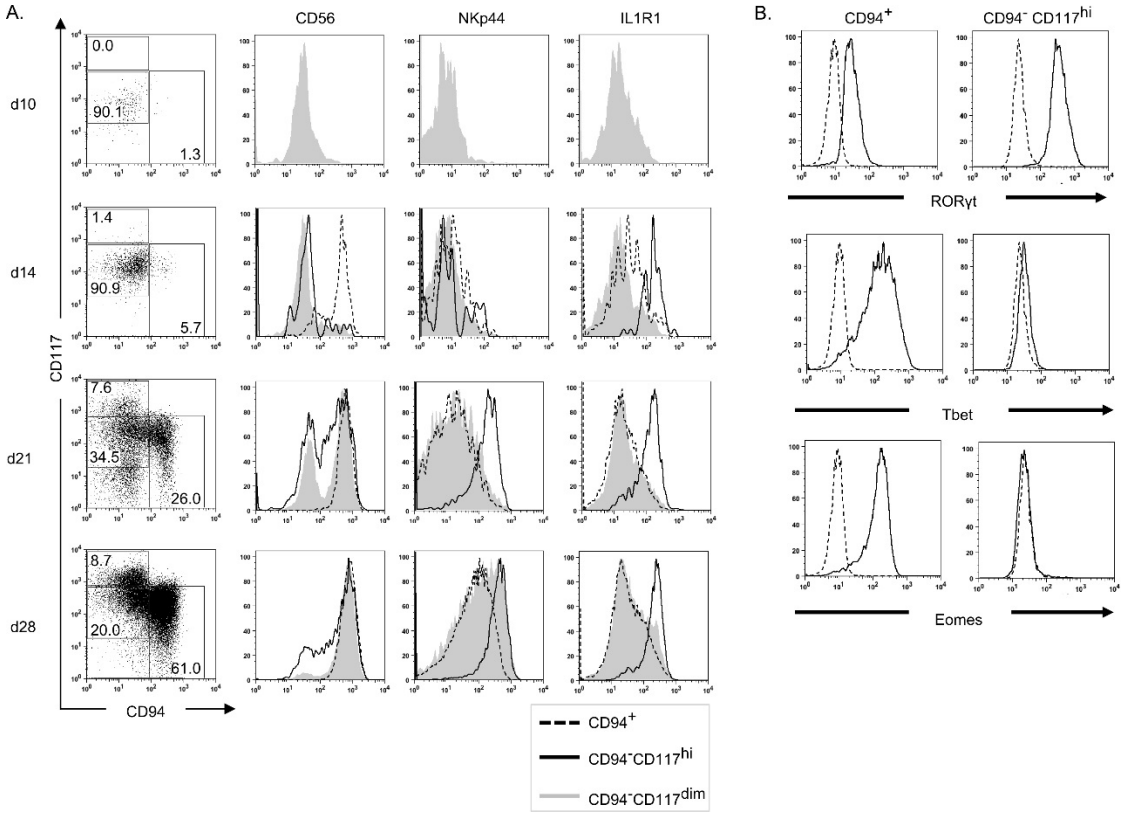

Figure 1: Generation of NK cells and ILC3 in vitro from CD34+ cord blood progenitors.

A) Flow cytometric analysis of CB differentiation cultures under NK/ILC supportive conditions. At day 10, 14, 21 and 28 cells were stained for CD117, CD94, CD56, NKp44 and IL1R1. All populations were gated for human CD45 expression or as indicated. Histograms showing CD56, NKp44 and IL1R1 are shown as an overlay of the populations as indicated; B) Intranuclear staining for RORγt, Tbet and Eomes is shown for the indicated populations. Gating was perfomed as in panel A. Marker expression (full line) is shown compared to an isotype control staining (dotted line). Representative analysis of 3 experiments are show at each time point.

After 14 days of culture, when the stage 3 population was most apparent, this population contained both IL1R1$^+$ and IL1R1$^-$ cells (Figure 2A). The IL1R1$^+$ stage 3 and IL1R1$^-$ stage 3 population were both negative for NKp44 expression. Within the IL1R1$^+$ stage 3 population, some CD56$^+$ cells could be detected (Figure 2A). Low levels of RORγt, Tbet and Eomes expression were detected on the protein level in the IL1R1$^-$ stage 3 population. In contrast, the IL1R1$^+$ stage 3 population expressed high levels of RORγt, and expression of Tbet or Eomes was borderline (Figure 2B).

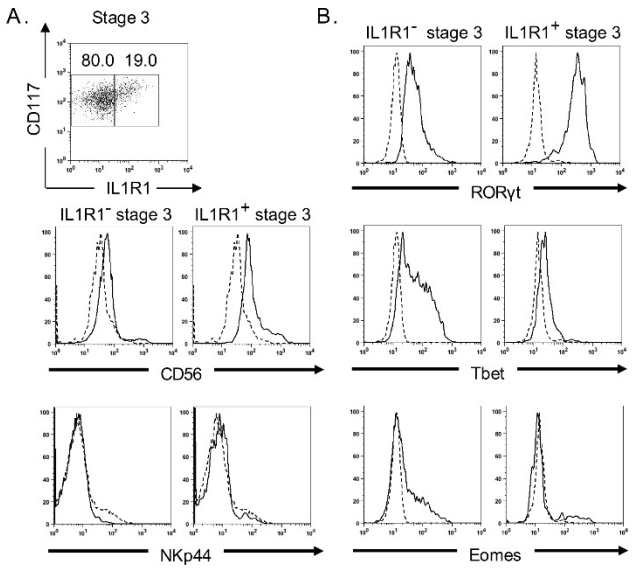

Figure 2: Stage 3 NK precursors contain a distinct IL1R1^{+} and IL1R1^{-} population.

A) Flow cytometric analysis of day 14 CD94^{-}CD117^{dim} stage 3 cells. Stage 3 cells were stained for IL1R1, CD56 and NKp44. All populations were gated for human CD45 expression or as indicated. Histograms show CD56 and NKp44 expression (full line). Expression is compared to an isotype control (dotted graph); B) Intranuclear staining for RORγt, Tbet and Eomes is shown for the indicated populations. Gating was perfomed as in panel A. Marker expression (full line) is shown compared to an isotype control staining (dotted line). Representative analysis of 3 experiments of each population are shown.

To confirm the data obtained by flow cytometry, expression levels of these transcription factors were assessed by qPCR. Herefore, CD94^{+} NK, CD94^{-}CD117^{hi} ILC3, IL1R1^{-} stage 3 and IL1R1^{+} stage 3 populations were isolated by FACS sorting on day 14 and day 21 of cord blood differentiation culture (Figure 3). As expected, NK cells expressed high levels of Eomes and Tbet, at both time points analyzed, while being devoid of RORC expression. (Figure 3). RORC was found highest in the ILC3 population, while this population was consistently negative for Eomes and Tbet.

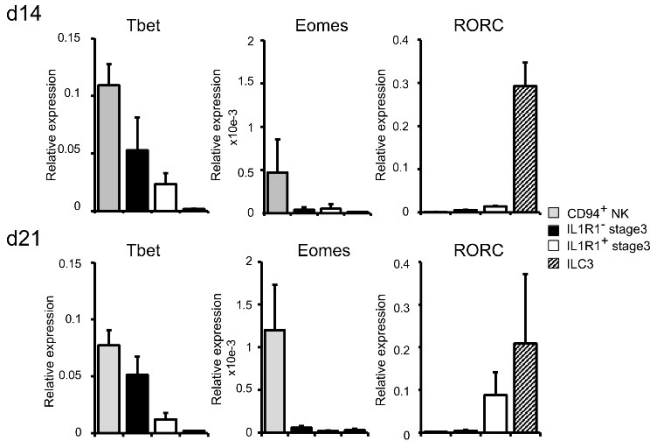

Figure 3: Expression of key transcription factors during innate lymphoid cell development.

Expression of Tbet, Eomes and RORC was analyzed in the indicated populations by qPCR analysis after 14 and 21 days of culture. Expression is shown relative to the mean of GAPDH and YWHAZ expression. Data represent the mean of 3 independent cord blood donors. Error bars indicate the standard deviation of the mean.

Both at day 14 and day 21 of culture, the IL1R1$^-$ stage 3 population expressed high levels of Tbet, but was negative for Eomes and RORC. In contrast, the IL1R1$^+$ stage 3 population expressed low levels of Tbet and RORC, but was negative for Eomes at day 14 of culture (Figure 3). 7 days later, expression of Tbet was decreased in comparison to the expression on day 14, but RORC expression increased in this IL1R1$^+$ stage 3 population (Figure 3). From these data it is clear that ILC3 and IL1R1$^+$ stage 3 cells are a distinct population, and while the latter population expresses RORC, some remnant expression of Tbet expression could be detected. This suggests that the IL1R1$^+$ stage is a transition stage from the IL1R1$^-$ stage 3 towards the ILC3 phenotype.

To address this hypothesis, IL1R1$^-$ and IL1R1$^+$ stage 3 precursors were isolated from day 14 cocultures through FACS sorting and recultured for up to 10 days (Figure 4). By the end of culture, IL1R1$^+$ precursors generated higher percentages of ILC3 (defined as CD94$^-$CD117^{hi}IL1R1$^+$NKp44$^+$) compared to IL1R1$^-$ precursors. The fraction of CD94$^+$ NK cells was similar between the progeny of both populations (Figure 4A). To address whether ILC3 and CD94$^+$ NK cells were generated with

altered kinetics, cultures were analyzed after 7 and 10 days of culture (Figure 4B). After 7 days, $CD94^+$ NK cells were most apparent in cultures initiated with $IL1R1^-$ stage 3 precursors, however, low percentages were also detectable in the $IL1R1^+$ stage 3 condition. By day 10, $CD94^+$ NK cells had formed from both populations to the same extent. This suggests a difference in kinetics of $CD94^+$ NK formation from different stage 3 subpopulations. Upon addressing ILC3 lineage differentiation, it was clear that $IL1R1^+$ stage 3 precursors had faster kinetics of ILC3 generation, and this fraction constituted a higher proportion of $CD45^+$ cells than the $IL1R1^-$ stage 3 precursor (Figure 4B).

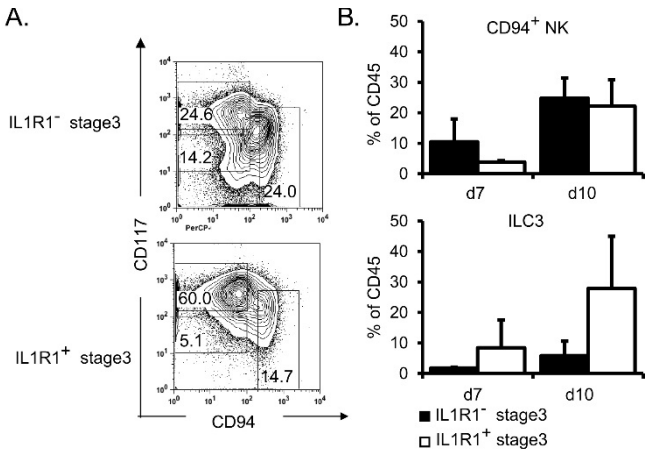

Figure 4: IL1R1$^+$ stage 3 precursors represent ILC3 primed precursors.

A) Flow cytometric analysis of IL1R1$^-$ stage 3 and IL1R1$^+$ stage 3 precursors sorted from d14 cocultures and recultured for 10 days of culture. Plots show CD45$^+$ gated fractions. Representative dot plots from the same donor are shown. B) Analysis of IL1R1$^-$ stage 3 and IL1R1$^+$ stage 3 progeny after 7 and 10 days of culture. Data are indicated as percentage of CD45$^+$ cells and represent the mean of 3 independent cord blood donors. Error bars indicate the standard deviation of the mean.

These data thus suggest that $IL1R1^-$ stage 3 cells readily differentiate into NK cells, or alternatively, differentiate into ILC3 through an $IL1R1^+$ stage 3 intermediate, which would explain the delayed kinetics of ILC3 generation.

Stimulation through the AHR influences lineage commitment of stage 3 precursors.

To address whether the development of ILC3 cells is influenced by AHR signaling, we added either the known high affinity AHR ligand 6-Formylindolo(3,2-b)carbazole (FICZ) or DMSO carrier to day 14 bulk cultures and analyzed the phenotype and cell number 4 days later (Figure 5). Phenotypically, induction of AHR signaling led to an increase of the ILC3 population (defined as $CD94^{-}CD117^{hi}IL1R1^{+}NKp44^{+}$), but showed a reduction of the $CD94^{+}$ NK population (Figure 5A). Addition of FICZ, did not significantly affect the percentage and absolute cell numbers of $CD45^{+}$ cells generated (Figure 5B, C). Although the differences are not significant, the $CD94^{+}$ NK cell population was decreased in the presence of FICZ after 4 days of culture, a similar result was found after 7 days of culture (Figure 5D and data not shown). Upon addition of FICZ both the percentage and absolute cell number of ILC3 significantly increased (Figure 5D, E). Within the stage 3 population, no significant changes could be detected upon addition of FICZ, however, the $IL1R1^{+}$ stage 3 precursors showed a tendency to decrease upon AHR stimulation (Figure 5D, E).

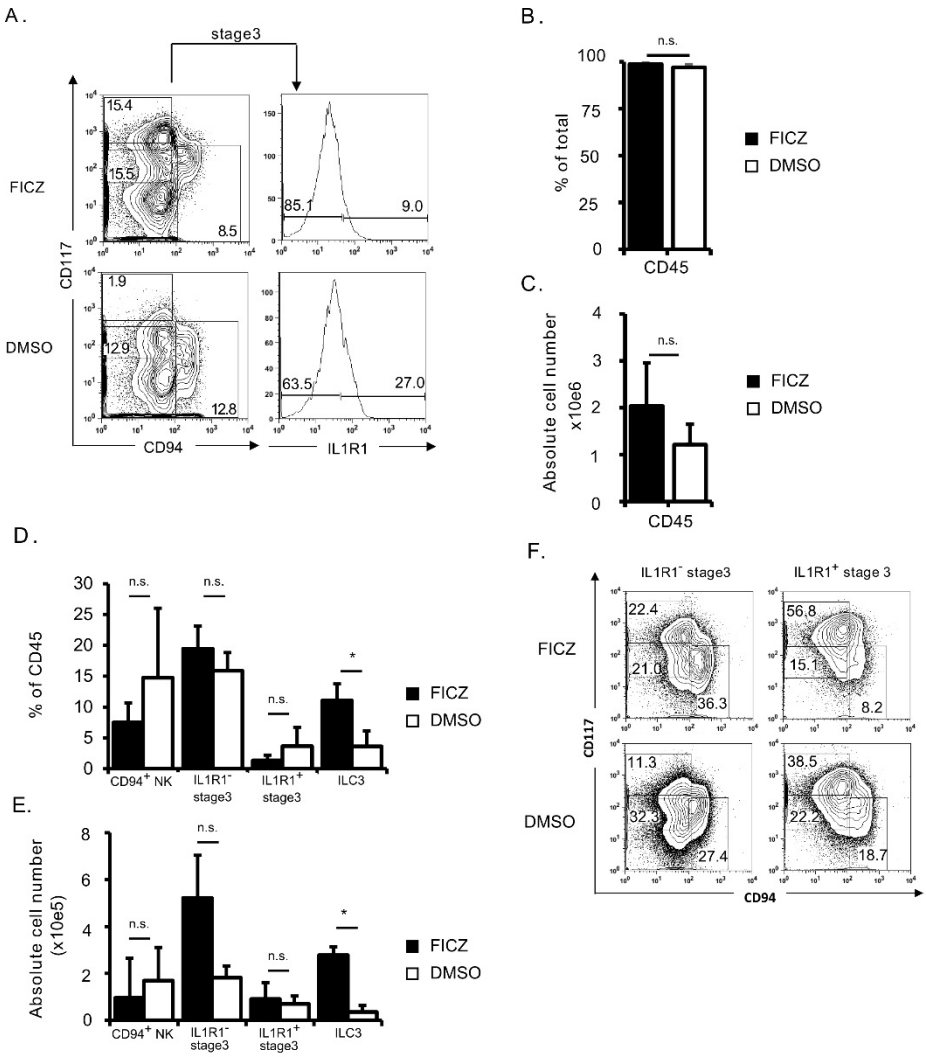

Figure 5: Stimulation of the aryl hydrocarbon receptor influences the lineage output of different stage 3 precursors.

A-E) Flow cytometric analysis of day 18 cocultures. Cultures were performed in the presence of the AHR agonist FICZ (300 nM) or DMSO carrier control from day 14 onwards. A) Plots show CD45$^+$ gated fractions. Histograms show marker expression within the stage 3 precursor population. B, C) Percentage and absolute number of CD45 in day 18 cocultures are shown. Data represent the mean of 3 independent cord blood donors. Error bars indicate the standard deviation of the mean D,E) Percentage and absolute number of the indicated populations in day 18 cocultures are shown. Percentages are shown as proportion of CD45$^+$ cells. Data represent the mean of 3 independent cord blood donors. Error bars indicate the standard deviation of the mean. F) Day 14 IL1R1$^+$ and IL1R1$^-$ stage 3 precursors were sorted and recultured for 10 days in the presence of the AHR agonist FICZ (300nM) or DMSO carrier control. Plots show CD45$^+$ gated fractions. Representative dot plots are shown from the same cord blood donor.

As the increase in ILC3 might reflect rapid differentiation of IL1R1^{+} stage 3 precursors towards ILC3, the influence of AHR stimulation on the lineage output of the two stage 3 subsets was addressed. Here fore, IL1R1^{-} stage 3 cells and IL1R1^{+} stage 3 cells were FACS sorted from day 14 cocultures. These stage 3 populations were subsequently allowed to differentiate for an additional 10 days in the presence of FICZ or DMSO carrier (Figure 5F). A subpopulation of IL1R1^{-} stage3 became a clear ILC3 population by day 10 (Figure 5F). The IL1R1^{+} stage 3 population gave rise to a population of ILC3 cells which constituted about 50% of CD45^{+} cells by the end of culture. The NK cell population generated from IL1R1^{+} stage 3 precursors was smaller than the population generated from IL1R1^{-} stage 3 precursors (Figure 5F). When the AHR was stimulated by addition of FICZ, development of NK cells from the IL1R1^{+} stage3 precursor was hampered, as evidenced by the decrease in the percentage of NK cells (Figure 5F). In contrast, NK cell development from IL1R1^{-} precursors was largely unaltered (Figure 5F).

In conclusion, AHR stimulation diverts differentiation of the IL1R1^{+} stage 3 precursor toward ILC3 development and hampers development of NK cells. In addition, AHR stimulation has no effect on the differentiation of IL1R1^{-} stage 3 precursor towards the NK lineage.

Discussion

To date the phenotypic intermediates of ILC differentiation have not been clearly described. Plasticity between different types of ILC has been proposed. However, while some reports have underscored this hypothesis, others have provided evidence against it. In the human system, it was shown in a report by Hughes *et al*. that IL-22 producing ILC3 found in human secondary lymphoid tissues express high levels of IL1R1 (11). In a recent report by Hughes *et al*., lineage plasticity of this IL1R1$^+$ ILC3 population was shown. In the absence of AHR signaling, ILC3 cells were able to differentiate towards NK cells, while stimulation of AHR blocked this differentiation. Also, IFNγ production by NK cells was markedly decreased after AHR stimulation(5). In a report by *Ahn et al.*, it was shown that in differentiation cultures initiated with human CD34$^+$ cord blood progenitors, ILC3 and NK cells could be distinguished by expression of LFA1 after 21 days of culture (6). At this timepoint, the stage 3 population already contained mature IL-22 producing ILC3 cells and IFNγ producing NK cells. Here, no plasticity of the ILC3 population could be detected. These findings suggest that late in culture, NK cells and ILC3 are terminally differentiated, and have lost their lineage plasticity.

We show here that innate lymphoid cells and conventional NK cells are efficiently generated in cord blood CD34 cultures as previously described (6, 12). In addition, we show the dynamics of IL1R1 expression during the development of ILC. During *in vitro* differentiation, NK cells and ILC3 are generated through a stage 3 NK cell precursor. After 14 days of culture, stage 3 NK cell precursors can be detected which express IL1R1 within this population. Both IL1R1$^-$ and IL1R1$^+$ stage 3 precursors have been shown to be present in secondary lymphoid tissues . These populations constituted about 20% and 80% of stage 3 cells, respectively (11). In culture, we found these IL1R1$^+$ stage 3 precursors to rapidly progress towards an ILC3 phenotype. Development of IL1R1$^-$ stage 3 cells seemed to preferentially proceed towards the NK cell lineage, however, these also gave rise to a small population of IL1R1$^+$ stage 3 cells and ILC3 later on (data not shown). These data are suggestive

for a model where IL1R1$^-$ stage 3 precursors develop into a IL1R1$^+$ stage 3 precursor, which subsequently gives rise to ILC3.

In the absence of AHR signaling, IL1R1$^+$ stage 3 cells were found to retain NK potential. Upon strong AHR signaling, NK cell development from the bipotent IL1R1$^+$ precursor was hampered. NK cell development from IL1R1$^-$ stage 3 precursors was not influenced by addition of an AHR agonist. These data are in contrast to the report of Ahn *et al*, where NK and ILC3 cells were not found to retain lineage plasticity after 21 days of culture. Our observations add to these findings that early in culture, NK and ILC lineages are diverting, but are not yet fully committed. In addition to the report by Ahn *et al*, it has been described that during development, murine NK cells do not develop through a stage of RORγt positivity (7) and that human IL-22 producing NK cells (ILC3), constitute a stable lineage, unable to generate NK cells (13). While these latter reports seem to be in sharp contrast with the reports of Hughes et al, where developmental plasticity of RORγt$^+$ ILC3 cells has been described (5), this might reflect differences in lineage commitment of ILC found in SLT.

As plasticity between NK and ILC3 is not detected *in vivo*, the bipotent nature of IL1R1$^+$ stage 3 cells might be an *in vitro* artifact and explain the absence of RORγt traced NK cells *in vivo*. During *in vivo* development of ILC3, signaling through the AHR has proven to be essential (14). In physiological locations of ILC3 development, levels of AHR ligand might be sufficiently high to induce ILC3 development from IL1R1$^+$ stage 3 cells and block NK cell development (15, 16).

In our study IL1R1$^+$ and IL1R1$^-$ stage 3 subsets were found to be different from mature NK and ILC3 populations in their transcription factor expression profile. IL1R1$^-$ stage 3 precursors express Tbet, low level of Eomes and do not upregulate of RORC. On the other hand IL1R1$^+$ stage 3 cells express low levels of Tbet, are negative for Eomes expression and upregulate expression of RORC. This suggests the former to be more NK primed precursors, while the latter seem primed towards the ILC3 lineage. This is reflected in the lineage output of these precursors, although, lineage plasticity can clearly be detected. While it has been established that RORγt is

essential for ILC3 development (17, 18) and that Tbet is needed to sustain NK cell populations (19), an essential role for Tbet in NKp46^{+} IL-22 producing ILC development has also been suggested (20). Moreover, Tbet expression was detected at high levels in this IL-22 producing subset. As we, and others (5, 6, 21), did not detect Tbet expression in ILC3, this might reflect differences between human and mouse. NK cells and ILC1 express high levels of Tbet, this thus seems to be a specific transcription factor for group1 ILC in the human system. Loss of Tbet expression and upregulation of RORγt expression in IL1R1^{+} stage 3 precursors thus suggests a gradual differentiation towards ILC3 cells. On the other hand, IL1R1^{-} stage 3 precursors maintain high levels of Tbet and are devoid of RORγt expression, which could explain their NK skewed lineage potential.

We here thus provide an explanation for differences in reports on ILC plasticity, by showing that during early development, ILC3 primed precursors retain NK potential. Within the stage 3 progenitor population, IL1R1^{-} progenitors maintain high expression of Tbet to form NK cell primed precursors, or they upregulate IL1R1 to become ILC3 primed precursors. Differentiation along the ILC3 pathway still holds lineage plasticity, and NK differentiation from this precursor can be blocked by strong stimulation of AHR early in development.

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7. Discussion of part II

The research described in part II aimed at elucidating the development of human innate lymphoid cells (ILC), by using an *in vitro* cord blood $CD34^+$ coculture system.

The first ILC family member that was discovered, was the NK cell in 1975 (1). About 30 years later, other subsets of innate lymphoid cells have been described (2-4). For years, it has been known that NK cells derive from a common lymphoid precursor (CLP), together with T and B cells (5). However, with the discovery of a whole new family of immune cells, it remained to be seen through which developmental stages these cells develop. It has been clearly shown that all ILC subsets derive from the CLP, however, their exact developmental pathways and the plasticity between different ILC subsets has remained matter of scientific debate.

In the research we have performed, we provide evidence that could explain the seemingly contradictive reports on ILC lineage plasticity. In addition, these insights in the development of ILC do not only offer valuable fundamental scientific data, but also allow for development of novel targeted therapies.

7.1 Do ILC hold lineage plasticity during development, and do environmental cues influence plasticity during differentiation?

The fact that all (innate) lymphoid lineages derive from a CLP in the adult has been established (5). However, whether a common ILC precursor exists remains a matter of debate. Also, whether there is plasticity between different types of ILC remains to be established.

Secondary lymphoid tissue derived precursors have been described to contain ILC3, which retain the ability to generate conventional NK cells (6). Stimulation of these ILC3 with IL-1β led to their expansion and caused an increase of IL-22 and AHR expression, while decreasing IFNγ production. Moreover, IL-1β signaling negatively influenced NK cell differentiation from this $IL1R1^+$ ILC3 (7). Recently, this $IL1R1^+$ IL-22 producing ILC3 was shown to be responsive to AHR stimulation. Stimulation of

AHR signaling promoted ILC3 differentiation and blocked their differentiation towards $CD94^+$ NK cells (6). Other reports, both in human and mouse, dispute this plasticity and describe a separate lineage of NK cells and ILC3 (8, 9). In the paper by Ahn *et al.* the $LFA1^+$ NK cells and $LFA1^+$ IL-22 producing ILC3 were shown to be stable lineages, and ILC3 were not able to generate NK cells or *vice versa*. These NK and ILC3 were obtained through *in vitro* differentiation of cord blood $CD34^+$ progenitors, and assessed after 21 days of culture (9). We reasoned, that these differences in plasticity might be due to differences in differentiation state of ILC3. We therefore determined whether this plasticity could be found in the stage 3 precursors that arose after 14 days of culture. We found the $CD94^-CD117^+$ stage 3 NK cell precursor population to be heterogeneous, this population could be subdivided on the basis of CD117 and IL1R1 expression. Within the stage 3 population, ILC3 can be distinguished based on the expression of high levels of CD117, NKp44 and IL1R1. These cells expressed RORγt and were devoid of Tbet and Eomes expression. This phenotype is compatible with previously published reports on human ILC (4, 6, 7, 9, 10). The true stage 3 population could be defined as $CD94^-CD117^{dim}$. Within this population both $IL1R1^-$ and $IL1R1^+$ populations could be detected. These cells have a distinct expression profile. $IL1R1^-$ stage3 precursors express high levels of Tbet, low levels of Eomes and do not upregulate of RORC. On the other hand $IL1R1^+$ stage 3 cells express low levels of Tbet, are negative for Eomes expression and upregulate expression of RORC. While similar $CD34^+$ cord blood progenitor differentiation cultures were previously described, we describe for the first time the kinetics of IL1R1 expression during the development of ILC (6, 11).

The data we obtained is suggestive for the presence of NK and ILC3 primed precursors. This is reflected in the lineage output of these precursors, although lineage plasticity can clearly be detected. In culture, the $IL1R1^-$ stage 3 population generated $IL1R1^+$ stage 3 cells and subsequently formed ILC3, albeit with low efficiency and delayed kinetics. These $IL1R1^-$ stage 3 cells mainly generated NK cells. On the other hand $IL1R1^+$ stage 3 cells mainly gave rise to ILC3 cells, but retained NK developmental potential.

An essential role of RORγt was described for ILC3 development (16, 17), while Tbet is essential for the maintenance of NK cells (18). A recent report showed that Tbet is highly expressed in $NKp46^+$ ILC22 and has an essential role in their development

(19). However, in the human system, such a role for Tbet was not detected, as Tbet expression was found to be limited to group1 ILC (NK and ILC1) (5, 6, 20).

Our expression data of these transcription factors in IL1R1$^-$ and IL1R1$^+$ stage 3 populations, in combination with the lineage potential of these cells, thus suggests a gradual differentiation towards ILC3 cells of IL1R1$^+$ stage 3, while IL1R1$^-$ stage 3 precursors show an NK skewed lineage potential.

We found that IL1R1$^+$ stage 3 cells still hold NK potential, however NK cell development was hampered and ILC3 development was stimulated upon strong AHR signaling by FICZ, a known AHR agonist (11). AHR stimulation had no apparent effect on the lineage output of IL1R1$^-$ stage 3 precursors. In the report of Ahn *et al*., NK and ILC3 from day 21 cocultures did not show lineage plasticity in the absence of AHR agonist (9). Using the same differentiation protocol, we show that the early stage 3 precursors still hold lineage plasticity. During murine NK cell development, no stage of RORγt positivity could be detected (7). In addition human IL-22 producing NK cells isolated from SLT, currently called ILC3, were unable to generate NK cells (12). The seemingly contradictive reports on developmental plasticity of RORγt$^+$ ILC3 might thus reflect differences in lineage commitment of ILC found in SLT. The fact that no RORC marked NK cells could be found in lineage tracing experiments, might merely reflect that in homeostasis sufficient AHR agonists are present in locations where ILC3 reside (8, 12, 13). This could prevent ILC3 lineage plasticity and block differentiation towards conventional NK cells. ILC3 plasticity might thus just be an *in vitro* artefact, induced by insufficient AHR agonist present in culture. It would be interesting to revisit the RORC lineage tracing model, to see under which conditions *in vivo* ILC3 reversion would occur.

7.3 How can knowledge of innate lymphoid cell development and function be translated to the clinic?

As described earlier, ILC have an important role in clearing gastrointestinal infections, in addition they have a role in preventing GVHD (14) and aid in restoring gut epithelia during inflammatory bowel disease (15) . On the other hand, they have a disease-

promoting role in cancer (16), psoriasis (17) and asthma (18). It is thus clear that specific ILC subtypes could be used or targeted *in vivo* in the treatment of certain diseases. To this end the development of different types of ILC could be skewed or their activation status could be altered. This could be achieved by administration of cytokines, chemical compounds, cytokine binding monoclonal antibodies, or addition of monoclonal antibodies that could block signaling through specific receptors.

Among the family of ILC, ILC3 were described to produce IL-22 (19) and IL-17 (20).

In the case of IL-17, a pro-inflammatory cytokine also produced by TH17 T cells and ILC3, evidence for a causal effect was found for inflammatory bowel disease (21, 22). Here, IL-17 induces and sustains an inflammatory response (23). A role for a TH17 response has also been described in mediating GVHD (24). In both of these diseases there seems to be a relation between the TH17 response and the TH1 response, the latter is often related to the disease etiology.

In the case of colon cancer, IL-17 has been described to be involved in promoting tumor development from colonic epithelial cells, and induces therapy resistance (25). Neutralizing IL-17A reduces the tumor load in mouse models and might thus provide a means for therapy.

In the case of psoriaris, the role of IL-17 has is not cleas. IL-17 can be detected in activated keratinocytes, however only IL-22 appears implicated in the disease (26).

A role for IL-17 in asthma has been suggested (reviewed by Chesné et al (27)), this role is probably restricted to a subtype of the disease, as anti IL-17 therapy showed no beneficial effect in a clinical study on subjects with severe to moderate asthma (28).

Further research is thus needed to link IL-17 to these diseases and to see whether IL-17 producing ILC are implied. Understanding the developmental pathways of these types of ILC could also provide a means to skew development or plasticity of IL-17 producing ILC3 towards IL-22 producing ILC3.

IL-22 signaling has been reported to play a role in several diseases and might provide a novel target for several therapies (reviewed by Sabat *et al.* (29)). Among these are GVHD (14), IBD (15, 30, 31), psoriasis (17) and asthma (18).

Interestingly, IL-22 seems to have a differential effect depending on the disease. In the case of psoriasis and tumors, the IL-22 signaling pathway seems to have a disease promoting role, while in the case of asthma and GVHD, IL-22 signaling seems to provide a protecting role. (32).

Involvement of IL-22 has been described in the development of gastrointestinal cancer (16). Here, a decrease in the expression of IL-22 binding protein, which strictly maintains IL-22 levels in homeostasis, leads to deregulation of STAT3 signalling. In colorectal cancer, deregulated STAT3 signalling was detected (33). Consequently, a specific IL-22 binding antibody, an IL-22 blocking monoclonal antibody or IL-22 binding protein could be administered for the treatment of these malignancies. A similar strategy could be used to treat psoriasis. Alternatively, the IL-23R could be blocked in the intestine, leading to a reduced activation of IL-22 producing innate lymphoid cells.

After allogeneic stem cell transplantation, it was shown that IL-22 producing ILC3 were eliminated through GVH T cell responses, which induced GVHD related intestinal toxicity (14). As IL-22 was shown to have a protective role for intestinal stem cells, It is thus clear that IL-22 signaling is also involved in this severe pathology. Development and function of ILC3 depends on AHR stimulation (34), thus AHR agonist stimulation of the limited number of ILC3 might provide a means to increase the number of IL-22 producing cells. This could circumvent the prolonged administration of IL-22 to these patients. One of these AHR agonists is FICZ, a tryptophan derivative (11).

In the intestine, IL-22 causes the intestinal crypts to bud, restoring the intestinal lining. In the case of Crohn's disease, IL-22 has been described to reconstitute goblet cells, increase production of antibacterial proteins and repair the epithelial barrier (30, 31). For these diseases, protease resistant IL-22 could be provided. Alternatively, as in the case of GVHD, administration of AHR agonists might provide a suitable means for the therapy of intestinal bowel disease (35, 36).

Conventional NK cells have an important function in the immune response against cancers. Interestingly, several cancers have been described to increase tryptophan metabolisation using tryptophan dioxygenase. This leads to increased production of kynurenine (Kyn). In this system signaling through AHR by Kyn has been shown to

influence the T cell antitumor responses (37). It was shown that stimulation of the AHR influences the balance of NK over ILC3 differentiation. This balance might promote cancer development in both ways: by limiting the differentiation of NK cells, which could provide antitumor effects, and by increasing the number of ILC3, which have been shown to have a cancer promoting effect through the production of IL-17 and IL-22. The developing tumor might thus not only act locally on the surrounding T cells and NK cells, but might influence the development of stage 3 NK cells in the secondary lymphoid tissue, through systemic release of AHR agonists. Compounds that influence these pathways may prove key in limiting tumor growth and stimulating anti-tumor responses.

Adoptive transfer of *in vitro* generated NK cells might provide a suitable therapy for several malignancies. Tumor cells evade the immune-surveillance provided by NK cells through several mechanisms: they up-regulate MHCI expression or they down-regulate the ligands for activating receptors. This causes endogenous cells to be incapable of killing the developing or established tumor. To this end, adoptive transfer of allogeneic NK cells or NK cell lines might prove a highly functional alternative, and this method has been successfully applied in patients (38, 39). However, the MHC mismatch might cause elimination of the transfused NK cells. In addition, expansion of NK cells *in vitro* might induce exhaustion (40), and freeze/thawing of NK cells hampers their function (41). Also the use of NK cell lines, which are often derived from malignantly transformed cells themselves, is questionable. As an alternative source of cells for allogeneic transfer, NK cells have been efficiently generated from several HSC sources, such as umbilical cord blood (UCB) (42) and bone marrow (BM) (43). These *in vitro* generated NK cells could then be directly used for transfusion.

A novel approach in NK cell immunotherapy is the retargeting of NK cells through transgenic expression of chimeric antigen receptors (CARs). These CARs consist of single-chain variable fragments (scFv) in combination with T-cell signaling domains, which enables their triggering upon recognition of antigens by this CAR (44). This increases tumor-NK cell interactions. This has been shown to improve cytotoxicity of NK cell lines against different antigens (45-50). This method could be applied to peripheral blood derived NK cells, NK cell lines or *in vitro* generated NK cells. Thus

combination of *in vitro* differentiation of NK cells with introduction of CARs into $CD34^+$ might provide an interesting source of NK cells for adoptive transfer.

As an alternative source, NK cells have been successfully generated from human embryonic stem cells and pluripotent stem cells. As protocols have been described to efficiently generate functional NK cells from PSC (51, 52). Sufficient numbers of NK cells might be obtained using the xeno-free protocol described by Knorr *et al*. (52). Upon addition of artificial antigen presenting cells to these cultures, the authors were able to show 100-1000fold expansion of the NK cells initially obtained (52).

Thus, in conclusion, while several options are possible to treat diseases in which ILC are involved, most are these have not been taken beyond proof of principle *in vitro* or in animal models.

8. References to the discussion of part II

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Publications

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Poster presentations

International Society for Experimental Hematology (ISEH) – 2014
In vitro human embryonic stem cell hematopoiesis mimics MYB independent yolk sac hematopoiesis.

Stijn Vanhee, Katrien De Mulder et al.

Oncopoint – 2014
cMYB expression during *in vitro* hematopoiesis from human embryonic stem cells
Stijn Vanhee, Katrien De Mulder et al.

Belgian hematological society (BHS) – 2014
cMYB expression during *in vitro* hematopoiesis from human embryonic stem cells
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International Society for Experimental Hematology (ISEH) – 2013
cMYB expression during human *in vitro* hematopoiesis
Stijn Vanhee, Yasmine Van Caeneghem et al.

Workshop on Innovative Mouse Models (IMM) – 2013
Transgenic human embryonic stem cell lines for the study of cMYB in human hematopoiesis
Stijn Vanhee, Katrien De Mulder, et al.

Belgian Hematological Society (BHS) – 2013
Establishement of transgenic human embryonic stem cell lines for the study of human hematopoiesis.
Stijn Vanhee, Katrien De Mulder, et al.

Wetenschapsdag – 2012
Human primitive type blood cells are generated independent of Notch signaling *in vitro*.
Stijn Vanhee, Katrien De Mulder, et al.

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Belgian Hematological Society (BHS) – 2012
Human primitive type blood cells are generated independent of Notch signaling *in vitro*.
Stijn Vanhee, Katrien De Mulder, et al.

Belgian Hematological Society (BHS) – 2011
Definition of hematopoietic precursor cells in an *in vitro* human embryonic stem cell hematopoietic differentiation model.
Stijn Vanhee, Imke Velghe, et al.